THE ABRAHAM FLEXNER LECTURES
SERIES NUMBER NINE

A STORY OF NUTRITIONAL RESEARCH

The Effect of Some Dietary Factors on Bones and the Nervous System

by
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PUBLISHED FOR VANDERBILT UNIVERSITY
by
THE WILLIAMS & WILKINS COMPANY
BALTIMORE
1950

Made in United States of America

PUBLISHED FOR VANDERBILT UNIVERSITY
BY
THE WILLIAMS & WILKINS COMPANY
COMPOSED AND PRINTED AT THE
WAVERLY PRESS, INC

FOREWORD

The Flexner lectures which comprise the present volume were originally to be given in the year 1941. Sir Edward Mellanby had already been invited to deliver them when war intervened and the arduous tasks imposed upon him as a result prevented his acceptance. Although at the time the postponement was regarded as one of the unfortunate sacrifices of war, it now appears to have been no sacrifice since the material described has been greatly extended during the interim and the hardships involved in doing research in England during the war gives it an interest which would have been lacking six years ago.

Sir Edward describes in these lectures a series of investigations which systematically developed from observations made early in his research career, observations which played a prominent part in the birth of the Science of Nutrition, and which have continued on and off over a period of thirty years. Although during much of this time other important duties have fallen to his lot, including the onerous task of stimulating and coördinating much of the medical research in Britain on behalf of the Government, the duration and continuity of the work described make the present lectures in a sense a scientific autobiography, which, apart from its peculiar value as such, should prove valuable to the neophyte in research as an example of patient, logical and step-wise development of the solution of a problem.

Although the text of the narrative itself does not reveal it, we who were privileged to know the Mellanbys read between the lines the constant encouragement and active assistance given by Lady Mellanby. Much credit must be given to her for the splendid work recorded herein.

THE ABRAHAM FLEXNER LECTURESHIP

The Abraham Flexner Lectureship was established November 22, 1927 in the School of Medicine of Vanderbilt University through the generosity of Mr. Bernard Flexner of New York City. An adequate endowment was provided to secure as lecturer at intervals of two years some eminent physician or scientist who has made a definite contribution in the field of medical science, or in some science allied with medicine, who would remain in residence for a period of two months and become associated with the teaching personnel and students of the school.

This Lectureship was created in recognition of the great service which Doctor Abraham Flexner, as Secretary of the General Education Board, rendered to medical education through the reorganization and development of the new School of Medicine of Vanderbilt University. Mr. Bernard Flexner wished in this way to permanently associate his brother's name with the institution which he had so generously helped to establish and improve.

The significant objective of the Flexner Lectureship was to contribute to the maintenance of the highest ideals and standards of scholarship in medical education and research. The Lectureship it was hoped would also be of value in the development of cultural interests in medical education and high ethical standards in medical practice.

The original plan included arrangements for the publication of the formal lectures in serial volumes by the University authorities in order to extend the influence and scientific value of the Lectureship.

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INTRODUCTION

The Origin of the Problems Discussed

In 1913 the Government of Great Britain established the Medical Research Committee, with the duty of advising the Government how best to spend on medical research £40,000 to £50,000 a year, a sum accumulated from the annual contribution of one penny by each person insured under the National Health Insurance Act of 1911. This was the beginning of the state's financial interest in the serious promotion of medical research, and indeed probably of any scientific research in Great Britain.

In 1920 the Committee became known as the Medical Research Council and was granted a Royal Charter of incorporation, under the general direction of a committee of the Privy Council. A grant-in-aid was provided by the Treasury; this subsidy has gradually increased with the passage of years and is now in the region of £600,000 (1947–48).

Soon after the formation of the Medical Research Committee the Great War of 1914–18 broke out and, while it might be imagined that this disaster would have ended the research enterprise, in reality it offered an opportunity, which was immediately grasped, to organise research on some of the special medical problems of war. What was gained by the advance of war medicine was for a time lost in other fields of research but in 1919, with the cessation of hostilities and a return to more normal conditions, the Government, influenced by the success of organised medical research in wartime, decided to endow the work on the broader and more stable basis referred to above.

It is interesting to see the lines along which the original Research Committee began its task, especially as this

course of lectures is a direct, if remote, outcome of the programme drawn up in 1913.* First, a list of major disabling diseases besetting the country at that time was prepared and those individuals who had shown a particular interest in any of these or were judged to be fitted for the task were asked to investigate one or more of the diseases from some specific angle. Among the items listed were tuberculosis, rheumatism (including acute rheumatic infections and chronic arthritis), rickets and oral sepsis.

The author was one of those invited to study the aetiology of rickets, a disease so common in Britain at that time, especially in the manufacturing towns of the north, that the grossest bone deformities were accepted without comment by the people, and the hypotheses and theories held by physicians and medical scientists as to the aetiology of the disease were legion. In parts of the USA the disease was also very common.

The problem of rickets which the writer was asked to investigate was the part processes of oxidation might play in the aetiology of the disease. It is not necessary to describe the many futile attempts made to study rickets from this particular angle, all of them directed in the first place to the experimental production of the disease in

* These facts are mentioned because, in the absence of large private endowments in Great Britain at that time, work of the type to be discussed in these lectures, involving as it does large and prolonged expenditure, could not have been undertaken without financial support from the state. It is a matter of great interest that other conditions have obtained in the USA, where a vast amount of medical research, often of the highest quality, was privately endowed, notably by the Rockefeller Foundation, at a time when there was no state endowment. In recent years, the state in the USA has begun to play an important part in such finance; in Britain, on the other hand, some substantial private endowments for medical research have now become available.

animals under controlled conditions. Since it was well known that rickets was commonly found in puppies, these animals were chosen for the experiments, which began in 1915. During the early stages of the work tests were made with diets of high specific dynamic action to see whether increasing the intensity of oxidation by this means affected bone formation. For this purpose puppies were given diets which contained large quantities of lean meat and, as a result, developed very deformed legs. Although the disease in these cases was osteoporosis as well as rickets, yet these experiments, by demonstrating what serious bone abnormalities could result from faulty diet, undoubtedly orientated the work towards the hypothesis that this might well be a primary factor in the aetiology of the disease. From this time onwards the investigations became more and more directed along dietetic and nutritional lines and the question of oxidation sank into the background. Simple diets were ultimately found which produced rickets in puppies and were compatible with good growth and health until severe rachitic changes developed (E. Mellanby, 1918, 1919, 1920, 1921).

There were two general results of this early experimental investigation, the main one being the discovery that rickets was due primarily to a deficiency in the diet of an antirachitic vitamin which was fat-soluble and was thought at the time to be vitamin A, or a substance closely allied to it in properties and distribution. The second result was the discovery that cereals had a rickets-producing effect and that the greater their consumption the more severe the disease, provided the diet was deficient in the antirachitic or calcifying vitamin. Now, of course, this was not the first ailment to be established as a deficiency disease. Goitre had been ascribed to a lack of iodine by Chatin in 1850. Chlorosis, a common malady in Europe in the early years of the present century, was

known to be curable by iron and could be regarded, therefore, as a deficiency disease. Beri-beri had been shown to have this aetiology by Eijkman in 1897 and scurvy had been similarly placed by the work of Holst and Frölich in 1907. In the USA and Britain, however, beri-beri and scurvy were both regarded as interesting possibilities for study rather than as practical problems of everyday life; but rickets, which was one of the great social diseases of western civilisation, was another matter and, soon after it was proved to be a deficiency disease, the importance of the quality rather than the quantity of the diet began to be recognised in both countries. This tendency for greater public interest in the ingredients of the food was also stimulated by the work of May Mellanby, who established the fact that the structure of teeth depended upon the type of diet eaten during their development and that in this case also the calcifying vitamin held a key position (M. Mellanby 1918–29). Moreover, it was shown that the better calcified the teeth the less liable were they to decay (M. Mellanby, 1923, 1934).

Another reason for the great public interest in the importance of quality of diet, which was first roused by this work, was the acute controversy which arose during the early period of its publication both in Britain and America. A number of workers in Glasgow led by Findlay and Noël Paton had found support for the view first advanced by Hansemann (1906) that rickets was due to 'domestication' and all that the word involves. A series of publications involving animal experimental work by Findlay (1908, 1915), Renton and Robertson (1916) and by Paton, Findlay and Watson (1918) had been supported by a large scale investigation of the social survey type (Ferguson 1918) in the belief that the main cause of rickets was hygienic and that it was largely due to 'inadequate air and exercise'. They not only refuted the

suggestion that a fat-soluble vitamin in the diet was essential for calcification of bone but they even discountenanced the idea that defective diet was in any way involved in the aetiology of rickets. In America also, Hess and Ungar (1920) tested the effect of a diet deficient in fat-soluble vitamin on children and failed to obtain any evidence that this vitamin was associated with rickets. In 1920 they wrote: "It is impossible to interpret the contrary conclusion which Mellanby came to as a result of his pioneer experiments on dogs or to accept the term fat-soluble vitamin as synonymous with antirachitic factor." Later work, as is now well known, revealed that the problem of bone calcification is a complicated process centring round a fat-soluble calcifying vitamin, so complicated indeed that there is little wonder that the early days of the deficiency theory of rickets were attended with so much controversy.

At the time of this early work on rickets (1916–20) only one fat-soluble vitamin was recognised, namely vitamin A, which had been discovered by McCollum and Davis in 1913. But little was then known of its chemistry or distribution and it was identified only by two biological tests: (1) its power to promote the growth of *young rats*, and (2) the fact that, in its absence from the diet, *rats* developed xerophthalmia, which could then be cured by the administration of the vitamin. To cut a long and difficult story short, it was ultimately found that the original vitamin A of McCollum and Davis was really a mixture of two fat-soluble vitamins—one of which retained the designation of vitamin A while the other, the calcifying or antirachitic vitamin, became known as vitamin D. The subject was later further complicated by the identification of at least two vitamins A (A_1 and A_2) and two main vitamins D (D_2 and D_3) and many other subsidiary ones, but this matter will not be

pursued here. Some consolation may come to those primarily interested in fat-soluble vitamins in that, difficult as it has been to follow their history and multiplication, it is child's play compared with the effort to keep in touch with the innumerable modern offspring of the old water-soluble companion vitamin, vitamin B.

Looking back over the years, it is interesting to note that, of the two biological properties which allowed the determination of the original vitamin A complex—growth promotion of young rats and anti-xerophthalmic action—the former was just as much due to the vitamin D as to the vitamin A moiety. A detailed discussion of the effect of vitamin A on bone growth in young animals can be found in Chapter VIII. As regards the action of vitamin D, the antirachitic part of the original vitamin A complex, this is now known to be essential for the proper growth of bones since, in its absence, calcium metabolism becomes abnormal, so that ultimately all bone growth stops. Thus, there was no more reason why the name 'vitamin A' should not have been given to what is now known as 'vitamin D' than that it should have been retained to designate the anti-xerophthalmic entity. It might have been less confusing if, as the A complex became separated, the terms A_1 and A_2 had been used instead of A and D.

The differentiation of the vitamin A complex into its constituents required the labour of a large number of investigators over several years (Med. Res. Coun. Spec. Rep. Ser. No. 167, 1932), and it was not until after 1924 that it became possible with certainty to give animals the calcifying vitamin, i.e. vitamin D, in the form of D_2, without also including the anti-xerophthalmic vitamin A. In that year Steenbock and Black (1924) showed that vegetable oils, devoid of fat-soluble vitamins, acquired the calcifying properties of vitamin D when exposed to ultra-violet radiation. This was an extension, via the

work of many others, of the fundamental discovery made by Huldschinsky (1919, 1920) that children could be cured of rickets by exposure of their skins to ultra-violet radiation. This aspect of the subject was further developed by the joint announcement of Rosenheim and Webster (1927) and of Windaus and Hess (1927) that ergosterol was the pro-vitamin D in fats and could be converted into the vitamin by exposure to ultra-violet radiation. This part of the story ended when vitamin D (calciferol) was prepared by Bourdillon and his colleagues (Askew, Bourdillon, Bruce, Jenkins and Webster, 1930).

The closer examination of the nutritional properties of vitamin A became possible when Euler and his colleagues (Euler, Euler and Hellström, 1928) found that carotene had the biological properties of the vitamin and could be converted to it in the body. Shortly after, Karrer obtained a highly concentrated preparation of vitamin A and determined its structure (Karrer, Morf and Schöpp, 1931); the effects of adding this substance in pure form to synthetic diets could then be studied in greater detail.

When the full account of the results obtained in the study of experimental rickets in puppies was published by the author in 1921, a special section at the end of the report was given to the description of three conditions which had been prominent in the experimental animals at one time or another and which could not be regarded as part of the rachitic syndrome itself. The first of these was headed: 'Susceptibility to Anaesthetics'; the second: 'Nervous Symptoms', and the third: 'Diminished resistance to Infection'. It was the second group, 'Nervous Symptoms', which attracted particular attention and, as the first three lectures of this series were devoted to an account of this part of the work and its subsequent development, it may be of interest to see what was written

in 1921 about the abnormal behaviour of some of the dogs in which rickets had been experimentally produced. This was as follows:

'The appearance of nervous symptoms in the puppies eating defective diets is common. (Note: 'defective diets' means diets which produced rickets in this investigation.) They can be classified into three main groups: (1) In animals confined to their kennels a common occurrence is that on being allowed freedom they are incapable of running straight. Often they run round and round in a small circle. At other times they behave as if intoxicated, starting off for a few steps in one direction, swaying in another direction and then falling over. This may be repeated again and again until the puppy comes to the conclusion that it cannot arrive at any one point it aims at and then it sits down. In both these cases it appears as if there were something wrong in the vestibular nerves or cerebellum.'

Then follows a description of paralysis of the hind legs and a short account of the tetany and convulsions which occasionally develop in rachitic animals and have attracted much attention. These subjects will not be discussed here, except to say that the paralysis is mainly due to a defective calcium-phosphorus metabolism and is closely related to rachitic changes in bone. It is still true, however, that there is no definite knowledge of the pathological basis of paralysis under these conditions.

It was to the subject of the abnormal behaviour of rachitic animals, involving great ataxia and incoördination of movement, that the attention of the author was drawn again and again until, finally, it became one of major interest to him and has, in a sporadic way, remained so ever since. The curious outcome of experiments designed to explain the reason for the incoördination will form the

theme of the earlier lectures. This work only became possible when it was established that the vitamin A part of the original complex, and not the antirachitic or vitamin D moiety, was the central nutritional factor in the study of this phenomenon (Mellanby, 1926).

The last two lectures will also deal with a subject referred to in the early publications on experimental rickets, namely, the rachitogenic or anti-calcifying effect of cereals. It is a curious fact that practically all attention in work on rickets since 1921 has been directed to the part played in calcification by the antirachitic vitamin D; the anti-calcifying action of cereals has not only been largely avoided as a subject of study, but the fact that cereals have any such specific effect on calcification was even officially denied at one time in the USA. Thus, the Council of Food, writing in the Journal of the American Medical Association for 3 July 1937 (p. 31), said: "It may be *concluded that there is no good evidence for the existence* ot a decalcifying factor in cereals, and that the hypothesis of the existence of such a factor is not needed to explain experimental results". It will be agreed that, if cereals have *such an action, indeed if they have any action on* bone calcification at all, then it must be a major problem of human nutrition, whether considered as a physiological, social or economic problem, since cereals form from 40% to 70% of the diet of most races of the world. It is for this reason that attention will be given to the subject in this course of lectures.

Having now sketched the background of the investigations and the conditions under which they were made, it may be well to give the titles of the lectures upon which the present volume is based and which were delivered at the Medical School of Vanderbilt University in March 1947 as the Abraham Flexner Lectures.

Vitamin A—Its Effect on Bone Growth and the Nervous System

Lecture I. Incoordination of movement due to nerve degeneration in vitamin A deficiency.

Lecture II. Bone dysplasia causing nerve degeneration in vitamin A deficiency.

Lecture III. Modelling and growth of bone—the influence of vitamin A on osteoblastic and osteoclastic activity.

Cereals and Calcification

Lecture IV. The anti-calcifying and rachitogenic action of cereals—their phytic acid content.

Lecture V. The action of phytic acid in the presence and absence of vitamin D in animals and man.

PART I

VITAMIN A DEFICIENCY AND INCOÖRDINATION OF MOVEMENT

Chapter I

INCOÖRDINATION IN YOUNG ANIMALS DUE TO VITAMIN A DEFICIENCY

By 1926, it had been established that the incoördination and ataxia of the rachitic animals referred to above was not due to a deficiency of the antirachitic vitamin D but to the absence of the vitamin A moiety of the original A complex of McCollum and Davis. This condition, unlike rickets, could not be prevented by the addition to the diet of irradiated vegetable oils. On the other hand, green vegetables which were regarded at that time as rich sources of the antixerophthalmic vitamin A, but poor in vitamin D, prevented ataxia but might not prevent rickets. Substances like fish liver oils and egg yolk containing vitamins A and D in sufficient amounts, prevented both conditions. Butter prevented the ataxic symptoms and improved calcification, but might not prevent rickets (Mellanby 1926).

In order to demonstrate the nature of the syndrome under consideration, a brief account will first be given of the behaviour of young animals of different species brought up on mixed diets of good nutritive value deficient in, but not completely devoid of, vitamin A and carotene (see Appendix I). It is important to realise that this is not a problem of a single species of animal, but one which is common to the young of all species so far examined, namely rabbits, dogs, ferrets, rats and chickens.

1. Behaviour in Vitamin A Deficiency

Rabbits

Young rabbits aged about 6 weeks grow (Fig. 1) for several months on a diet deficient in vitamin A and caro-

tene, such as that described on page 195. After about two months, however, they begin to show signs of abnormality, the order of appearance of which varies, though slight stiffness of the legs, especially of the hind legs, is generally the first noticed. Xerophthalmia is seen after three to six months of the experimental diet; a

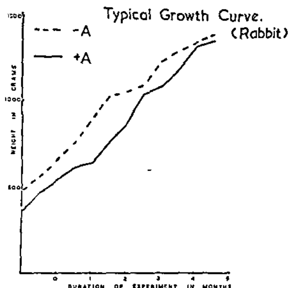


FIG 1 Weight curves of two litter-mate rabbits (aged 6 weeks at beginning of experiment). One received the −A diet only (see Appendix); the other had the same diet supplemented by vitamin A (mammalian liver oil). These curves are typical of those obtained in this investigation.

dull patch or band appears on the cornea, usually running midway between and parallel to the eyelids, i.e. in the part most exposed, and the pupillary reactions are found to be sluggish. Abnormal head movements often develop quickly after this, if they have not already begun. The animal moves its head in a curious and characteristic way as if it did not know its position in relation to the

rest of the body; the movements are usually up and down but sometimes from side to side, and when at rest the head is often askew, with ears awry. In the later stages some of the head movements may be due to ocular defects and obscured vision. The rabbit often moves about as if suffering from alcoholic intoxication. It may turn its head permanently to left or right, go round in circles, or wobble from side to side, and in extreme cases even fall over either sideways or backwards, sometimes being unable to regain the usual posture.

At no time is there any definite general paralysis, but the animals appear heavy on their legs and show less spontaneous movement, both in the cage and outside, and in advanced cases often refuse to move at all. A casual examination makes it clear that the coördinating mechanism, both of the head and of the body generally, is defective, and in severe cases it is obvious that the animals are also blind and probably deaf.

Dogs

If the vitamin A- and carotene-deficient diet described in Appendix I (1b) is given to puppies from the age of 6–8 weeks, incoördination of movement begins in about 2–3 months' time and may be severe by the end of 5 months. Usually the animals retain their appetite throughout the experimental period of up to 6 months and increase in weight at about the same rate as controls eating food of the same composition, but with the addition of vitamin A (see Fig. 2).

Behaviour varies somewhat from animal to animal, especially in the earlier stages of the deficiency. Although usually vigorous in their movements, the young dogs are unable to run straight and may lurch from side to side, or even fall over. Sometimes they circle for long periods or they may run about sniffing vigorously, licking

the ground and attempting to eat anything they come across. A noticeable early feature in most cases is that they cannot fix their attention for any length of time; unlike normal animals, they will not follow an individual for more than a few seconds, but tend to wander off, lurching in all directions. Generally they hold their heads in abnormal positions, usually on one side with the nose

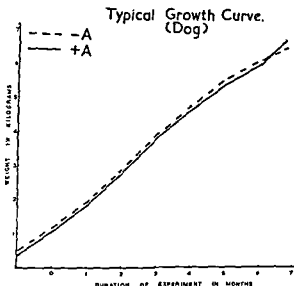


FIG 2. Weight curves of two litter-mate dogs (aged 6 weeks at beginning of experiment). One received the −A diet only (see Appendix); the other had the same diet supplemented by vitamin A (mammalian liver oil). These curves are typical of those obtained in this investigation

turned upwards and ears awry. In the later stages, stiffness, especially of the hind legs, greatly reduces their activity and, if the experiments are protracted, they may move very little, if at all. Later, the animals become deaf and sometimes blind; they sleep a lot, and it may be necessary to touch them in order to draw their attention to the presence even of food. To what extent their im-

mobility is due, on the one hand, to loss of hearing, sense of position (especially of the head), and possibly loss of or impaired vision, or, on the other hand, to a loss of muscle function, is not clear, but probably all these factors are involved.

Ferrets

It has been easy to produce in ferrets changes similar to those seen in rabbits and dogs. When fed on diets deficient in vitamin A and carotene (see Appendix I (1c)), they begin to develop abnormalities at varying times, usually after 3 to 8 weeks. First the fur becomes ruffled and they lose their sleek appearance. Soon after this it will be noticed that they sleep more during daylight hours than the control animals. They are still active, however, but their movements are less well-controlled and they hold their heads as though they were lost in space. At feeding time they become excited at the approach of food, but nevertheless may have difficulty in finding it after it has been placed in the cage. From this stage the abnormalities develop quickly, and xerophthalmia, advanced head movements, circling, severe incoördination and apparent deafness are soon evident. One change noticed in some ferrets but not in other species of animals studied is a 'reversed' or 'retreating' walk.

Rats

Rats beginning diet 43 (described in Appendix I (1d)) when 3 to 5 weeks old, remain well and breed provided that sufficient vitamin A or carotene is supplied, but the rate of growth and the final weight are subnormal. If no additional vitamin A or carotene is given, they still grow for about 4 months, but sooner or later during this period they develop certain abnormalities. Like ferrets, they lose their sleek look and the fur becomes ruffled; stiff-

ness of the hind legs and hunching of the back soon follows. They may hold their heads permanently to one side, and incoördination may develop, but it is not usually as severe as in the other species used in this investigation. The eyes are often affected with xerophthalmia, sometimes so badly that the whole cornea may be involved, and keratomalacia and hypopyon may result.

If, from about the twelfth day after parturition, mothers and young are given the diet 43 without additional vitamin A or carotene, then the latter grow for about 2 to 2½ months, after which their appetite is reduced and they become gradually very weak. These animals show eye changes and are incoördinate, but it is difficult to say to what degree because of their increasing weakness.

Diet 43 contains much oatmeal and therefore contains a trace of carotene. If, however, synthetic diet of purified substances (similar to that of Wolbach and Bessey, 1941) free from both vitamin A and carotene is given to the mother and the young from the twelfth day after parturition, then in the experience of this laboratory the weight of the young increases, though at a gradually decreasing rate, for about 1 or 1½ months; it is by then usually stationary and very soon afterwards begins to fall. Weakness and reduced activity, due to this loss of weight and inanition, develop so quickly and intensely that incoordination is difficult to recognise.

Chickens

Abnormalities similar to those described in dogs, rabbits, ferrets and rats can also be produced in young birds by vitamin A- and carotene-deficient diets (Appendix I (1c)). They develop a ruffled appearance; their feathers droop and lose their normal silky look. The birds become incoördinate and stagger, then use their wings in an effort to maintain an even balance, but this

only adds to their difficulties as the wings increase their movement and thereby their incoördination. When the eyes of these chickens develop xerophthalmia, the entire corneas quickly become infected and, as in the rat and dog, may rupture.

2. Factors Affecting Development of the Syndrome

Other points may be mentioned about the development of abnormalities in all these species.

(a) *Stores of vitamin A in the body.* If an animal which has been reared on a diet containing large quantities of vitamin A or carotene is put on a deficient experimental diet, abnormalities due to the deficiency will not develop as quickly as in an animal whose early diet has contained less of the vitamin or its precursor. In the former case, a large amount of vitamin A is stored in the liver and must be utilised before signs and symptoms of the later deficiency appear.

(b) *Age.* Young animals are more quickly and intensely affected by the deficient diet than older animals. Dogs given a diet deficient in vitamin A and carotene from the age of 1½ to 2 months usually become incoördinate after a period of 2 to 5 months, but if the diet is not begun until they are 4 to 6 months old, then the condition may not develop for 8 to 12 months. In adults first receiving the deficient diet at the age of about 30 months incoordination may not be seen for 15 or more months and even then although definite, it is never as severe as in the badly affected younger animals.

The Problem

Here then is the problem to be solved: Why and in what way do young animals of all kinds tested—dogs, rabbits, rats, ferrets and chickens—when fed on diets deficient

in vitamin A and carotene develop incoördination of movement, especially of the head, and in some cases become completely deaf and blind? Clearly the first line of attack was to examine the nervous systems to see whether there were any special lesions associated with and accounting for the abnormal behaviour described. This will be discussed in the following chapter.

Before continuing the story of the investigation, it might be well to give some idea of the quantities of vitamin A which can prevent the abnormal changes in puppies. 100 i.u. of vitamin A per day is too small a dose to prevent all the incoördination changes. 1000 i.u. give complete protection. Let it be assumed that 500 i.u. would be sufficient. Now 5 i.u. of vitamin A weigh 1 γ, so that 500 i.u. weigh 100 $\gamma = \frac{1}{10}$ mg. The average daily intake of food of an experimental puppy weighs, when dry, about 150 g. Thus one part of vitamin A in about $1\frac{1}{2}$ million parts of food is sufficient to prevent all defect. It will be realised from these figures how very powerful is this substance in directing proper development and function of young animals. The question is—how does this minute quantity of substance prevent the sequelae above mentioned?

Note: (1) From the beginning of Chapter II onwards reference to diets containing or deficient in vitamin A implies the presence or deficiency, as the case may be, of vitamin A itself, its precursor carotene, or any preparation containing either of these substances.

(2) For the sake of brevity, the term +A animal (dog, rabbit, rat, ferret or bird) will often be used to denote one brought up on a diet containing vitamin A or carotene, and the term −A animal for one brought up on a diet deficient in, but not entirely devoid of, these substances.

Chapter II

NERVE DEGENERATION IN VITAMIN A DEFICIENCY

The purpose of the present chapter is to give a brief account of the widespread degenerative changes in the nervous systems—both central and peripheral—which are produced especially in young animals when fed on diets deficient in vitamin A. It is these defects which cause the abnormal behaviour noted in the foregoing chapter. (See also Mellanby, 1926, 1931, 1933, 1934, a & b, 1935). Before describing the distribution of these changes, a short account will be given of the type of degeneration found, and especially of early lesions in the myelin sheath.

Histological methods for demonstrating demyelination changes, especially those involving the use of osmic acid, are apt to give unreliable results unless used critically: therefore most of the experiments were repeated many times and nerves of +A and −A animals were carried through and examined together. The tissues from each animal were often stained by several methods (including Marchi and modifications, Scharlach R and Nile blue sulphate). Weigert preparations were made when degeneration was severe (see Appendix).

Most of the nerves which are described as degenerated in the present chapter, show typical Wallerian degeneration. However, in some part of this investigation, especially in the work on xerophthalmia, the early degenerative changes demonstrated by Marchi's method assumed a *position of importance and it is necessary to discuss this* kind of appearance more fully. In the early cases of the *deficiency only a few scattered fibres in a nerve may be*

grossly changed, and in very early cases none may show typical Wallerian degeneration but the myelin, although still surrounding the axis cylinder, may be altered in chemical composition so that, when stained by Marchi's method, it becomes dark brown or black. In longitudinal sections such fibres may appear wavy and swollen. In transverse section, they appear as whole or broken rings, giving what has been called annular degeneration (Mellanby, 1934b). Similar rings were also seen 24 to 48 hours after cutting nerves. It was felt that these appearances represented a real chemical change of a degenerative type, but some neurologists seem to regard them with suspicion as artefacts. It was a matter of some importance, therefore, to see what experience other workers had had of the early degenerative changes affecting the myelin sheath and whether there was any substantial support for the view that annular degeneration found in the experimental animals is a stage prior to typical Wallerian degeneration and corresponds to the early degenerative changes, including the annular type, found after the cutting of the nerve fibres.

In this connexion, Bucy (1928) described experiments in which he cut nerves and found that the fibres passed through an early stage of abnormality, i.e. within the first two to four days, when they appeared to be wavy; the myelin sheath was intact, but swollen and rather granular, and assumed a dark brownish or black appearance with the osmic acid of Marchi's stain. On the clinical side, similar appearances were described by Kinnier Wilson (1913) as being a myelin change commonly seen in the spinal roots of patients who had died of pellagra. He referred to this appearance of nerve fibres as 'Bandstreifen', and the condition under discussion may at that time, therefore, have been more recognised by German than by British and American writers.

Culley (1927), in an investigation of polyneuritis in a

series of fowls, found that the myelin sheath became swollen and showed progressive disintegration, leading to severe fragmentation of the myelin sheath without demonstrable effects on the axis cylinder, and he also related these myelin changes to early Wallerian degeneration.

The subject of nerve reaction to harmful influences other than cutting was studied by Speidel (1935), who investigated the effect of such irritating stimuli as heat, chemicals and pressure on the nerves of frog tadpoles. As all his observations were made by direct microscopical examination of individual nerve fibres in the transparent tails of the intact living organisms, his technique did not involve the ordinary methods of fixing and staining. Speidel concluded that the changes produced by nerve irritation (including pressure) bore a marked resemblance to the early changes that characterized trophic (Wallerian) degeneration after nerve section, which, he said, included swelling of the fibres, formation of vacuoles which tended to separate the axis cylinder from the myelin sheath, and discontinuity of the sheath. Speidel also reported the effect of partial or total starvation, noting among other things, that the myelin sheath might become discontinuous without disintegration of the axis cylinder. If food was made available at this stage, then repair took place and the myelin in the sheath became continuous again.

In view of the present work and that of the other investigators quoted, it would not be surprising if nerve fibres showing these early anatomical changes resulting from injury should also show a chemical abnormality as revealed by Marchi and other stains. The recovery of anatomical continuity and other indications of repair noted by Speidel support the idea that rapid chemical recovery might also take place in these early stages.

It was suggested (Mellanby, 1934b) that, under the

experimental conditions used, when only annular degeneration had developed, it was likely that the administration of vitamin A or carotene would bring about rapid recovery to normal, thus indicating that annular degeneration was unlike that in which degenerative changes had proceeded to the typical Wallerian appearance with disintegrated myelin and axis cylinders.

It must be remembered that, when the work reported in this chapter was carried out, there was no knowledge as to the cause of the lesion. Now that it is realised that most, or possibly all, of the degeneration is due to pressure either on the ganglia or on the nerves themselves, it is obvious that here is a phenomenon about which even today little is known, since but few studies seem to have been made on nerve injury due to slowly developing pressure. Nerve degenerative changes produced under such conditions must be of much slower onset and of a more chronic kind than, for instance, the changes which follow the severing of the nerve. It would be of interest to know whether slowly developing pressure on a nerve or its ganglion causes a more prolonged stage of the earlier reaction than cutting the nerve. If such were the case, it might explain why annular degeneration, brought out by osmic acid, can be more easily demonstrated in this work than would be expected. While annular degeneration has been discussed at length because it is a subject of special interest in the present work, it must be remembered that typical Wallerian degeneration was the most common condition in which the nerve degeneration described below was found.

1. The Rabbit

(a) *Central Nervous System*

A series of drawings made from sections of the spinal cord and brain stem of a rabbit which had been on a

carotene and vitamin A-deficient diet for 28 weeks, showing typical degenerative changes, is seen in Figs. 4 to 13. These extend from the lumbar region of the cord, segment IV (Fig. 4) up to a section through the superior corpora quadragemina (Fig. 13). The positions of the main sensory and motor tracts in the rabbit's spinal

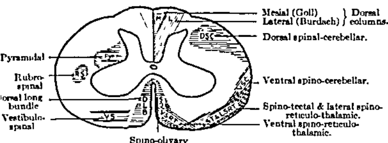


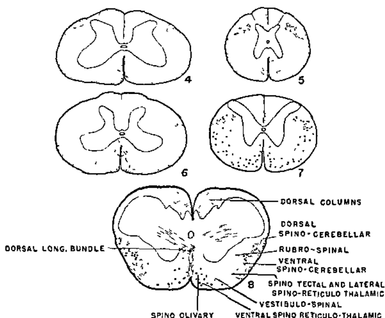
FIG. 3. Diagrammatic representation of the positions of the main tracts in a rabbit's spinal cord.

cord are shown in Fig. 3. The following points are brought out in the figures:

The following *ascending tracts* show degeneration:
- (a) The dorsal columns (Goll and Burdach) of the cord and the mesial and lateral fillets of the brain stem. The latter may include degenerated fibres from the second neurone of the auditory path.
- (b) The dorsal spino-cerebellar tract of the spinal cord which can be traced through the medulla to the restiform body and inferior cerebellar peduncle.
- (c) The lateral and ventral spino-reticulo-thalamic tracts.
- (d) The ventral spino-cerebellar tract which passes to the cerebellum via the superior cerebellar peduncle.
- (e) The spino-olivary tract (brain stem).
- (f) The ascending fibres of the dorsal longitudinal bundle which connects the vestibular nuclei with the 3rd, 4th and 6th nuclei (brain stem).

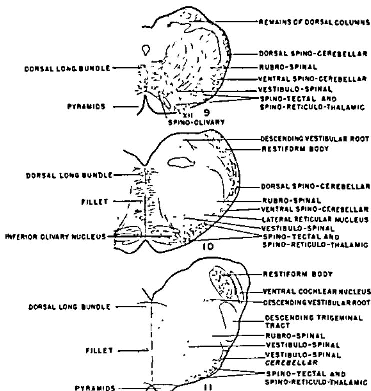
In the *descending tracts* degeneration is found in:
- (a) The rubro-spinal tract. Degeneration is most clearly seen in the mid-brain where the fibres decussate and in the medulla.

(b) The *vestibulo-spinal tract.*
(c) The dorsal longitudinal bundle. These fibres can only be *regarded as descending fibres at levels below the vestibular* nuclei.



FIGS 4–8. Drawings illustrating degeneration in the spinal cord and brain stem of a rabbit on a –A diet for 28 weeks.
Black dots represent degenerating fibres as revealed by Marchi method; circles represent fibres which have completely degenerated
FIG. 4. Lumbar segment IV
FIG 5. Thoracic segment VII
FIG. 6. Cervical segment VI.
FIG 7. Cervical segment II.
FIG. 8. Medulla, pyramidal decussation.

The main motor descending tract, namely the crossed pyramidal—there is no direct pyramidal tract in the rabbit—*is usually free* from degenerated fibres, although in advanced cases a few are occasionally found. It is also



Figs. 9–11. Drawings illustrating degeneration in the brain stem of a rabbit on a –A diet for 28 weeks.

Black dots represent degenerating fibres as revealed by Marchi method; circles represent fibres which have completely degenerated and disappeared.

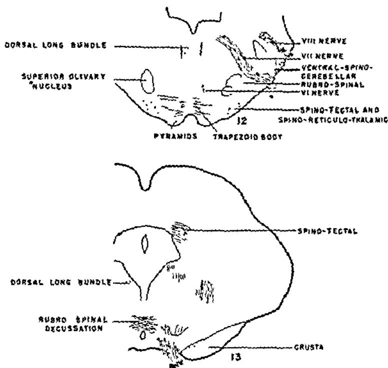
Fig. 9. Medulla, higher part of sensory decussation.

Fig. 10. Medulla, level of inferior olivary nucleus and 4th ventricle.

Fig. 11. Section between medulla and pons.

interesting to note in connexion with the obvious impairment of equilibrium in these rabbits that all degenerated descending fibres so far identified are connected with the vestibular or cerebellar systems. Degenerated

fibres are also seen in the intra-medullary portion of some of the cranial nerves, especially the VIIIth and Vth.



Figs. 12 and 13. Drawings illustrating degeneration in the brain stem of a rabbit on a −A diet for 28 weeks.
Black dots represent degenerating fibres as revealed by Marchi method.
Fig. 12. Level of VIIIth and VIIth nerves.
Fig. 13. Mid brain, level of superior corpora quadrigemina.
Note: The reduced number of degenerated fibres at these levels.

Probably the most outstanding feature of the distribution of the degenerative changes noted is their prominence in the medulla and cervical regions of the spinal cord. In the lumbar region of the cord the number of degenerated fibres is small and, above the level of the 8th cranial

nerve, there is also a great reduction in degenerating fibres.

(b) *Peripheral Nervous System*

The investigation was extended to the peripheral nerves. As abnormal head movements were always a prominent feature of vitamin A deficiency, the vestibular and cochlear divisions of the VIIIth nerve were first examined (Plate I, a and b), and it was seen that both suffered greatly, the cochlear division being more affected than the vestibular.

Degenerating fibres were also found in the optic nerves. This point will be discussed in Chapters III and VI. The other sensory nerve of the eye—the ophthalmic division of the Vth—is liable to undergo great destructive changes, but this also will be discussed later in relation to xerophthalmia (p. 47), one of the more common manifestations of vitamin A and carotene deficiency. Other afferent fibres of the trigeminal nerve such as the inferior dental nerve supplying the teeth and dental tissues are apt to become demyelinated.

Although the sensory nerves of the head in young rabbits are greatly affected by vitamin A deficiency, the motor cranial nerves, apart possibly from a few fibres occasionally found to be degenerated in the IIIrd nerve—a predominantly efferent nerve—are normal even when the main sensory nerves show great demyelination changes.

In view of the almost clear-cut division between the susceptibility of the sensory and motor cranial nerves to degenerative changes, it was a matter of interest to see whether other afferent and efferent nerves behaved similarly under these dietetic conditions. The likelihood of this being so was increased by the distribution of degeneration in the fibres of the spinal cord, referred to above, where it was recorded that the ascending fibres

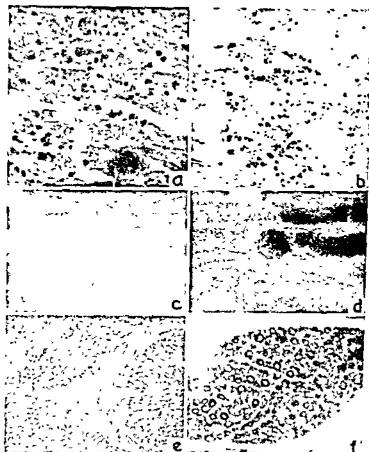


PLATE I

(a) Photomicrograph (× 325) of the vestibular nerve of a −A rabbit. (Staining by Marchi method.) Much degeneration.

(b) Photomicrograph (× 182) of the cochlear nerve of a −A rabbit (Staining by Marchi method.) Much degeneration.

(c) and (d) Photomicrographs (× 274) of dorsal root fibres of two litter mate rabbits of the same age. (Staining by osmic acid method described by Cajal.)

(c) +A diet Normal.

(d) −A diet. Fibres degenerating.

(e) and (f) Photomicrographs (× 182) of spinal roots (cervical VI) of a −A rabbit. (Staining by Marchi method.)

(e) Ventral root No degeneration.

(f) Dorsal root. Much annular degeneration.

were greatly affected, whereas the descending and motor fibres largely escaped destructive changes. Examination of the sciatic nerve also revealed degenerated fibres which in some experiments were very numerous. Since this nerve contains both efferent and afferent fibres it seemed likely, on the analogy of the cranial nerves, that the afferent fibres would degenerate and that the motor fibres would escape. To test this point, examinations were made of ventral and dorsal roots and it was found that, whilst the ventral (motor) roots were usually free from myelin degeneration, there was always some, and often much, degeneration in the dorsal (see Plate I, c–f). This relative susceptibility of the dorsal as compared with the ventral roots was also found in pellagrins (Wilson, 1913) and in A-deficient rats (Zimmerman, 1933).

It was expected that the vagus nerve would be similarly affected and that its afferent fibres would suffer while its motor fibres would escape harm. In the author's experience, however, this was not the case in dogs and rabbits. Examinations were made of the vagus nerves of many severely affected animals, and in no instance were degenerative changes seen. Zimmerman (1933), however, found some degeneration of this nerve in four out of eight A-deficient rats, while in all there was extensive degeneration of the medullary sheaths of the brachial plexes and sciatic nerves.

This differentiation between afferent and efferent nerves was difficult to explain; nevertheless it was thought at the time that the degenerative changes might be due to a neurotoxin acting on the nervous system affecting one type of nerve more than another.

(c) *Nerve Cells*

If it were true that the nerve degeneration was due to a neurotoxin, as at one time seemed possible, the formation or action of which was conditioned by the absence from

the body of vitamin A and carotene, then, in the absence of vitamin A, nerve cells might be expected to show the primary (toxic) degeneration described by Marinesco. So-called secondary or traumatic degeneration of the nerve cells is due to injury of the axon and includes the changes in the chromophile elements first described by Nissl (1892). In primary degeneration, as pointed out by Van Gehuchten (1897 and 1900), the chromophile particles (Nissl bodies) do not, as a rule, undergo true chromolysis. Marinesco, on the other hand, says that, when the lesions are primary, chromolysis commences in the periphery of the nerve cells and spreads towards the centre, whereas in secondary degeneration due to trauma the chromolysis begins in the centre and spreads to the periphery.

It is impossible in the experiments described in this work to classify all the degenerative changes according to one or other of these types. Many cells tend to fall into line with the primary or toxic group, whilst others show changes which appear to be typical of the secondary or traumatic variety. Often changes of both types may be present in the same group of nerve cells. Real chromolysis in the sense used by Nissl has been found, especially in the medulla, but often the particles lose their discrete character and are in a powdery and lightly staining form, the parapyknomorphic change of Nissl. At times the Nissl bodies may be aggregated into clumps and in other cases they may be so altered that the cell takes on a more intense, even stain. In addition to the changes in the cytoplasm, the nucleus and the nucleolus are often affected; the nucleus may be swollen, or shrunken, and eccentrically placed. At other times it is finely granular instead of clear.

It was recognised that there were normally differences in distribution of the Nissl granules of large and small cells, especially in the dorsal root ganglion. All the nerve

cell changes described here, however, refer to regular differences observed between those of the +A and −A animals, the sections of which were prepared and examined side by side (Mellanby 1935).

Dorsal root ganglia. Both the large and the small cells in these ganglia of −A animals are often abnormal. The Nissl granules in the main body of the cell are seen to be powdery and there is a concentration of granules round either the nucleus or the outer edge. Sometimes eccentric nuclei are seen (Plate IIb). The nuclei of the capsule or, according to Ramon y Cajal (1928), the subcapsule, have sometimes proliferated around degenerate cells, giving by Nissl's method a picture akin to what has been called 'neuronophagia' in the cells of the central nervous system (Plate IIa).

Gasserian ganglion. Degenerative changes are often seen, including eccentric nuclei, proliferation of the nuclei of the cell capsule, powdery granules and 'piling' of the granules around the nucleus and at the outer edge of the cell.

Clarke's column and other dorsal horn cells. The widespread degeneration found in the cerebellar tracts had suggested that these cells would be severely affected. Changes were found, but not as commonly as in the dorsal root ganglia. In advanced cases, however, some cells seem to disappear, leaving only a shell behind. In less severe cases neuronophagia (Plate II, c & d), loss of definition of granules, and eccentric and slightly granular nuclei are the general findings.

Other nerve cells. Degenerative changes are found in many other groups of nerve cells, including nuclei gracilis and cuneatus, the lateral reticular nucleus, the nucleus solitarius, nucleus ambiguus, vestibular nuclei (lateral and medial vestibular nuclei), descending 5th nucleus, red nucleus, and Purkinje cells of the cerebellum (which are often very badly affected (Plate II, e & f)).

Cells which appear generally to escape degenerative changes include lateral horn cells, ventral horn cells, hypoglossal nucleus, facial nerve nucleus, abducens and accessory 6th nucleus and oculomotor nucleus. Indeed, it is rare to find any significant degeneration in purely motor cells.

It will be noticed that nerve cells on the afferent side, especially in the spinal cord, suffer most. In the mid-brain, however, the red nucleus, dentate nucleus and sometimes the 3rd nucleus occasionally show some abnormality (Mellanby, 1935).

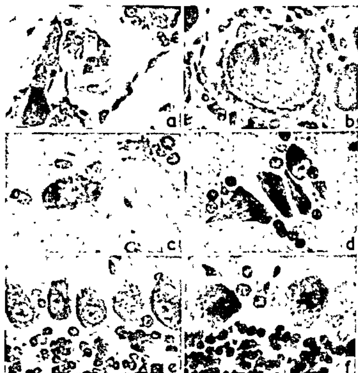


PLATE II

(a) and (b) Photomicrographs (× 490) of cells from the dorsal root ganglion of a −A rabbit.

(a) Corroded cell of Cajal.

(b) Large cell with eccentric nucleus and concentration of granules around the outer edge.

(c) and (d) Photomicrographs (× 490) of Clarke's column cells of two litter mate rabbits of the same age.

(c) +A diet (cabbage). Cells probably normal.

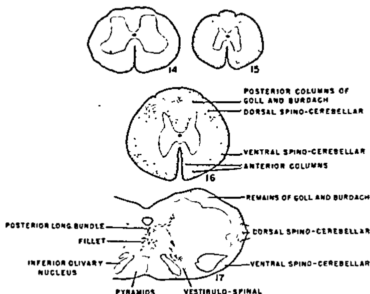
(d) −A diet. Cells with eccentric nuclei, loss of granules and neuronophagia.

(e) and (f) Photomicrographs (× 490) showing Purkinje cells (cerebellum) of two litter mate rabbits of the same age.

(e) +A diet (Mammalian liver oil). Cells probably normal.

(f) −A diet. Cells reduced in number; loss of differential staining.

The survey showed that as a rule the distribution of degenerative changes in the nerve cells corresponded to that seen in the fibres of the central nervous system.



FIGS 14–17 Drawings illustrating degeneration in spinal cord and brain stem of a dog on a −A diet for 13 weeks

Black dots represent degenerating fibres as revealed by Marchi method, circles represent fibres which have completely degenerated and disappeared.

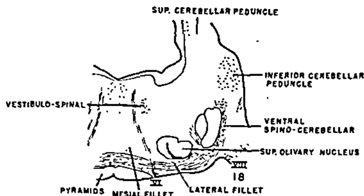
FIG 14 Lumbar segment IV.
FIG 15 Thoracic segment VII
FIG 16 Cervical segment I.
FIG 17 Medulla, level of sensory decussation

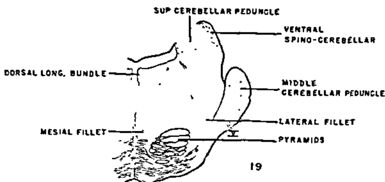
2. THE DOG

(a) *Central Nervous System*

As in the rabbit, the ascending fibres in the cord of −A dogs are very much more affected than the descending fibres (Figs. 14–19). Thus it is the dorsal and ventral spino-cerebellar tracts and the columns of Goll and Burdach that pre-eminently show degenerative changes. Also affected are ascending fibres in the ventral column.

Among the descending fibres commonly showing degeneration are the dorsal longitudinal bundle and the vestibulo-spinal tract.





FIGS. 18 AND 19. Drawings illustrating degeneration in the brain stem of a dog on a −A diet for 13 weeks.
Black dots represent degenerating fibres as revealed by Marchi method; circles represent fibres which have completely degenerated and disappeared.
FIG. 18. Lower part of pons.
FIG. 19. Upper part of pons. *Note:* Great reduction in number of degenerating fibres at this level.

Degenerating fibres of the two spino-cerebellar tracts can be traced into the cerebellum, the ventral tract via the superior cerebellar peduncle and the dorsal tract via the restiform body and the inferior cerebellar peduncle. The distribution, especially in the dorsal columns, varies in different experimental dogs. Usually

only a few fibres in the dorsal columns of the lumbar and thoracic segments are degenerated, but many are so affected in these tracts in the cervical region of the cord and in the lower regions of the brain stem. The second neurone of this series is also affected, for some, although not many, fibres of the fillet are degenerated (see Figs. 17 and 18).

It is rare to find any degeneration in the upper neurone motor tract (crossed pyramidal) and there is little or none in the brain at a higher level than the red nucleus.

(b) *Peripheral Nervous System*

(I) CRANIAL

1st Nerve (*Olfactory*). As the 1st nerve in passing through the cribriform plate is broken up into individual or small groups of fibres, histological methods were difficult to apply. Examination was therefore made of the surface of the olfactory lobe where the small nerve bundles enter the brain. As was expected from the vigorous sniffing of the deficient animals, degenerated fibres were a prominent feature in this region, whilst the olfactory lobe of the animal receiving vitamin A remained normal.

IInd Nerve (*Optic*). The optic nerves, really a part of the central nervous system itself, show severe degenerative changes (Plate III a & b) as the result of vitamin A deficiency and this may even progress to complete blindness; in one experiment where the deficient diet was fed for two years or so, all the fibres of the optic nerve were destroyed.

Vth Nerve (*Trigeminal*). Degeneration may be severe, and in animals in which the vitamin A deficiency has been maintained for a long period, destruction of most of the fibres may result. By examining both Marchi and Weigert-Pal preparations, all stages, from early annular degeneration (see p. 53) to the actual loss of fibres, may be traced. As in the rabbit, it is the first branch of the Vth nerve which shows the greatest changes, but degeneration has also been found in the second and to a less extent in the

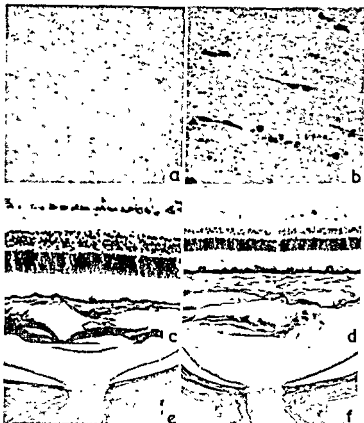


PLATE III

(a) and (b) Photomicrographs (× 182) of the optic chiasmas of two litter mate dogs of the same age. (Marchi stain.)
(a) +A diet. Normal.
(b) −A diet Much degeneration.
(c) and (d) Photomicrographs (× 65) of retinas of two litter mate rabbits of the same age
(c) +A diet (cabbage).
(d) −A diet.
Note: Retina is thinner, and cells of ganglion and inner nuclear layers are reduced in number and less intensely stained in (d) than in (c).
(e) and (f) Photomicrographs (× 4) of optic nerves and retinas of two litter mate dogs of the same age.
(e) +A diet.
(f) −A diet.
Note: Papilloedema in (f).

third division (M. Mellanby and King, 1934). For further reference to this problem see Chapter IV.

VIIIth Nerve (Auditory). This nerve shows the most severe changes of any of the peripheral nerves, the cochlear division being regularly more affected than the vestibular.

Other Cranial Nerves (IIIrd, IVth, VIth, VIIth, IXth, Xth, XIth and XIIth). It is rare to find degeneration of these nerves, though some may have a few degenerated or degenerating fibres in cases of severe vitamin A deficiency.

This completes the survey of cranial nerve degeneration in the dog, and it will be seen that practically all the afferent fibres, like those of the rabbit, are liable to destruction in vitamin A deficiency, though the vagus in both animals appears to remain intact.

(II) SPINAL

This sharp differentiation between afferent and efferent fibres is seen again when we examine the spinal roots, for it is the dorsal roots (afferent fibres) which degenerate, whilst the ventral roots remain normal. This is the usual result, at least in the earlier weeks of the experiment, though occasionally, when the deficient feeding period has been prolonged, a few fibres of the ventral root, which indeed may also be afferent, are occasionally affected, but the degeneration of the dorsal is always much greater than anything seen in the ventral roots. The sciatic nerve also shows changes, and from the distribution seen in the spinal roots it is likely that here again it is the afferent fibres which degenerate.

(c) *Nerve Cells*

The nerve cells of dogs affected by vitamin A deficiency are in the main the same as those shown to degenerate in the rabbit including the dorsal root ganglia and the

Gasserian ganglia, Clarke's column cells, Purkinje cells, and cells of the dentate nucleus. Here again are found such changes as powdering of Nissl granules, eccentricity of the nucleus and neuronophagia.

This survey of the distribution of degenerated fibres and nerve cells of the central and peripheral nervous system in −A rabbits and dogs being completed, it may be well to consider what deductions can be made from the evidence. Let us briefly consider the general position of the work at the time these investigations on the degree, nature, and distribution of the degeneration of nerve cells and of the myelin degenerative changes in the central and peripheral nervous system were made. Two questions presented themselves for answer:

1. Could the nerve degeneration be regarded as explaining satisfactorily the abnormal behaviour of the animals suffering from vitamin A deficiency?
2. Did the degenerative changes, either by their distribution or by their character, throw any light on the processes whereby the vitamin deficiency brought them about?

As regards the first question, it can be said with some assurance that the nerve degeneration did explain in a general way the abnormal behaviour of the animals. The ataxia could be accounted for by the degeneration of the vestibular division of the VIIIth nerve and by the widespread degeneration of cerebellar fibres and nerve cells. Deafness followed the early and severe degeneration of fibres in the cochlear nerve and defective vision of different degrees resulted from similar changes in the optic nerve. Undoubtedly closer study might reveal much more of significance in regard to the curious nerve lesions of these animals and their abnormal behaviour. It might be possible, for instance, to determine more accurately the real functions of the dorsal and ventral

spino-cerebellar tracts. For the present purpose, however, it is sufficient to say that the abnormal behaviour of animals suffering from vitamin A and carotene deficiency can be correlated with those degenerative changes in the central and peripheral nervous systems, which can be easily detected by histological methods.

The second of the above questions, namely, whether the degenerative lesions threw light on the mechanism whereby vitamin A deficiency produced them, was much more difficult to answer. It must be remembered that, when these nerve lesions were being mapped out, the author thought that they were probably due to a neurotoxin acting in the absence of vitamin A. That explains why a close study was made of the views of Marinesco (1909), Van Gehuchten (1897 and 1900) and Ramon y Cajal (1928) on the *differences in degenerative* changes in nerve cells when they were poisoned by a toxin (primary degeneration) and when the nerve fibres were injured (secondary degeneration). It was seen above (page 32) that the nerve cell changes appeared mixed in type, sometimes being of a primary and sometimes of a secondary nature. Careful consideration was at one time given to this aspect of the work and it was concluded that established knowledge at that time of the respective reactions of nerve cells to toxins and trauma did not offer sufficient aid to the solution of the problem as to whether the effective lesion was one primarily affecting the nerve cell or its conducting fibre (Mellanby, 1935).

Reference to the discussions of the experimental results published up to this time will show other difficulties of interpretation which prevented them from being fitted into any scheme which seemed reasonable. For instance, it was not found possible to produce nerve lesions in adults comparable in severity with those which developed in young animals; nor was it easy to understand how a

neurotoxin could pick out afferent from efferent nerve cells and having done so leave some afferent nerves, such as the vagus, unaffected. Again, why were the lesions so much more common in the lower brain stem and cervical region of the cord than in other places? Because of these and other difficulties this particular line of investigation was discontinued and attention was given to allied problems as described in the next chapter in the hope that light might be thrown on the relation between vitamin A deficiency and nerve degeneration.

Chapter III

ATTEMPTS TO DEVELOP THE PROBLEM

Several years intervened between the work described in Chapter II, showing the distribution of nerve and nerve cell degeneration produced in young animals by vitamin A deficiency, and the beginning of the research to be discussed in Chapter IV and the following chapters. This interval was partly due to the demands of other work, but mainly to the fact that a number of investigations undertaken with the object of carrying the problem a stage further, although leading to new knowledge, failed in their *primary* object, namely, the discovery why vitamin A deficiency resulted in nerve degeneration. The present chapter will deal with two of these investigations, since they concern a part of the subject of wide interest. First, a short account will be given of a study of the nerves and tissues of the eye in vitamin A deficiency, which it was hoped might lead to an explanation of the effects of this vitamin, and secondly, the search for a suspected neurotoxin in cereals whose action was conditioned by vitamin A deficiency will be described. In reading this chapter it would be well to remember that its object is not primarily to set out the latest available knowledge on these subjects, but to give a more or less chronological account of part of a long-term investigation. It is true, however, that but little fundamental information has been added to either subject in recent years. Where advances which affect the problems under consideration have been made, these are recorded and briefly discussed.

It was disconcerting to be faced with the fact that two of the main lesions produced in young animals up to 1926

by diets deficient in vitamin A were so dissimilar, namely:

(1) Hyperplasia and/or metaplasia of epithelium resulting in the formation of stratified epithelium with keratinization changes. The hyperplasia varies in degree and may be great or absent. Epithelia so affected include that of the alimentary canal and its glands, the respiratory tract, the genito-urinary tract and the eye and para-ocular glands. Often the affected epithelium is a focus of infection and in the eye at least may be a place of origin of an infective invasion of the surrounding tissue (Mori, 1922: Wolbach and Howe, 1925: Goldblatt and Benischek, 1927).

(2) Degenerative changes of certain medullated nerves in both the central and peripheral nervous systems of *rabbits* and *dogs* (Mellanby, 1926, 1931, 1933, 1935 and 1937) and *rats* (Zimmerman, 1933), (Irving and Richards, 1936).

These two different morbid changes did not appear to have any relation to one another. On the other hand, since the chief causative agent in both was the same, it seemed worth while to make an attempt to correlate the phenomena.

In retrospect it appears that the first of these characteristics of vitamin A deficiency, namely, changes in the epithelium and its possible invasion by micro-organisms, was over-emphasised, although all investigators on the subject would not agree with this statement. For instance, Wolbach and Howe (1925), who first described in detail the histological changes of epithelium in A deficiency, regarded the production of keratinizing metaplasia as its main primary effect. This was probably due to the fact that they and, indeed, the majority of experimenters up to this time, had made their observations on young rats, the epithelia of which are specially sensitive to deficiency of vitamin A. When these animals are brought up on synthetic diets composed of pure foodstuffs, such as were

used by most of the earlier workers, epithelial hyperplasia is practically constant and, in many of the places in which the epithelium suffers this change, local foci of infection often develop. On the other hand, it is relatively rare, even under experimental conditions of vitamin A deficiency, to find epithelial hyperplasia and metaplasia in other animals, such as the dog and rabbit. When they do occur, they are usually found in the eye in the form of xerophthalmia, and both the rabbit and the dog develop this condition. Xerophthalmia is, in fact, a specific reaction to vitamin A deficiency (McCollum and Simmonds, 1917), and, in the early stages at least, usually heals up at once, on administration of the vitamin. In human beings also, and especially in children (Bloch, 1924), xerophthalmia is known to develop when vitamin A deficiency is pronounced. Blackfan and Wolbach (1933) have described cases of A deficiency in infants where, in addition to the eye, many other places were found in which epithelial hyperplasia had developed. The exact relationship between epithelial hyperplasia, metaplasia and the onset of infection is not known with accuracy, except that it is generally recognised that the epithelial change is primary and is followed by the bacterial infection. Wolbach (1937) thinks the local infection of epithelium which has become keratinized is more or less fortuitous, and is due to the plugs of desquamated epithelial cells in ducts, bronchi and trachea, providing a suitable culture medium for bacterial growth. It is doubtful, however, whether this explanation covers the development of eye infection in xerophthalmia, especially in the dog. In this animal, it will be found on histological examination that, instead of there being hyperplasia of the corneal epithelium, there are often large areas where the thickness of the epithelium is reduced to one layer of apparently normal cells and there is a tendency for the cells lying immediately on top

of it to become keratinized. It is of interest to note that in the dog, where this characteristic epithelial condition is found, xerophthalmia is often associated, almost as soon as it develops, with an infective invasion of the substantia propria of the cornea (Mellanby, 1933). This may lead quickly to ulceration and hypopyon, a development which is more common in the dog than in other animals investigated.

Although the view is now generally held that keratinization of the conjunctival and corneal epithelium is a primary change, to which the inflammatory reaction is secondary and indeed not necessarily a result of keratinization, it is only right to add that other views of the mechanism whereby vitamin A deficiency leads to xerophthalmia have been held by various workers. Mori (1922), who was a pioneer observer in this field of epithelial change in A deficiency, regarded xerophthalmia as due to desiccation following the loss of secretion of the lachrymal glands and the ulceration of the cornea as resulting from secondary infection by micro-organisms. The main fact which has been advanced against this hypothesis is that lachrymation often persists after xerophthalmia has been established. Moreover, Wolbach and Howe (1925) pointed out that keratinization of corneal and conjunctival epithelium may occur without appreciable atrophy of the para-ocular glands. Findlay (1925) suggested that the most important factor in the onset of xerophthalmia in fat-soluble vitamin deficiency was a loss of or decrease in the lysozyme of the lachrymal secretion. Yudkin and Lambert (1923) thought that focal inflammatory lesions of the conjunctiva were the primary change and that keratinization was secondary.

Although, therefore, there had been much investigation and speculation on xerosis and the subsequent invasion of the cornea and eye by microörganisms, no account had been taken of the possibility that this condition might be

in some way related to the loss of neurotrophic control or some other protective mechanism against injury which would result from a pathological condition of the sensory nerve of the orbit. An investigation to test such a possible relationship seemed to be justified, since as was shown in Chapter II, it was known that the first division of the Vth nerve, i.e. the ophthalmic division, does, on some occasions at least, show degenerative changes in A deficiency. A short account will now be given of the work which was directed to the study of this point.

I. A Study of the Nerves and Tissues of the Eye in Vitamin A Deficiency

(a) *Xerophthalmia and the Vth nerve*

That the Vth nerve exerts a trophic control over the conjunctiva and cornea in man has been recognised by surgeons ever since the surgical removal of the Gasserian ganglion for trigeminal neuralgia was introduced. One of the most feared complications of this operation is the development of keratitis neuroparalytica. In an attempt to avoid this, ophthalmic surgeons usually try to protect the eyeball by sewing the eyelids together after the operation. The same phenomenon is sometimes seen when herpes zoster attacks the Gasserian ganglion, and here again resort is often made to the increased protection of the eye by the same means. It is clear, therefore, that conditions which interfere with the sensory nerve supply to the cornea are liable to be followed by infection, and because of this it is usually held that the afferent nerve supply to this tissue exerts some neurotrophic control.

EXPERIMENTAL RESULTS

Although lesions have been found in the eyes of A-deficient rabbits, dogs, rats, chickens and ferrets, histologi-

cal examination has been confined in this work mainly to rabbits and dogs.

Rabbits brought up from the age of 8–10 weeks onwards on diets deficient in vitamin A were killed at various periods after the development of xerophthalmia. The first sign of the disease in these animals is a small dull area or film running parallel to the eyelids. At the onset this may be present one day but gone the next, reappearing soon afterwards and gradually increasing in size and thickness. Periodically this mass may disappear, possibly being removed mechanically by the eyelids, leaving only a roughened surface on which the film soon recurs, until finally most or all of the epithelium may be damaged and covered by a thick opaque film.

Serial sections were made of the cornea of many of these rabbits, some of which were killed 24 hours after the first signs of xerophthalmia were noticed, some when the condition had become very severe and others with the disease in intermediate stages. Sections of the first division of the trigeminal nerve, taken both peripherally and centrally to the Gasserian ganglion, were found to contain degenerated or degenerating fibres, the number varying according to the severity of the condition. Only a few degenerated nerve fibres occurred in the earliest cases but, when the disease was severe, many fibres showed advanced and typical Wallerian degeneration. Control rabbits in each family on the same basal diet, but with the addition of some food containing carotene, showed no degenerative lesions either in the cornea or in the sensory nerve, provided sufficient of the supplement was given (*Plate IV*).

The following is a summary of the results obtained. Twenty-seven rabbits not receiving vitamin A or carotene developed xerophthalmia in one or both eyes. In twenty-three cases the condition was accompanied by degenerative changes in the ophthalmic division of the corresponding

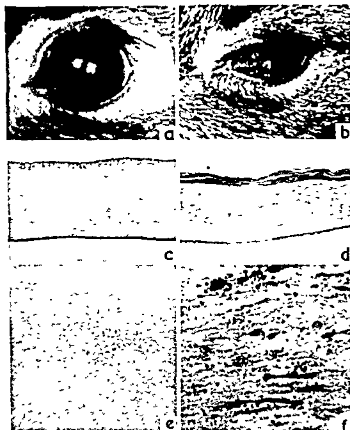


PLATE IV

Photographs and photomicrographs of the eye, cornea (× 65) and ophthalmic division of the trigeminal nerve (× 182) of litter mate rabbits of the same age.

(a), (c) and (e) +A diet.
(b), (d) and (f) −A diet.

Note. Xerophthalmia (b), keratinizing epithelium (d) and associated myelin degeneration (f) in the −A rabbit as compared with the normal appearance of eye (a), cornea (c) and nerve (e) in the +A rabbit.

Vth nerve. One animal in which xerophthalmic changes were found in only one eye showed degenerative lesions in the nerves to both eyes, whilst in another animal showing similar distribution of xerophthalmia both nerves were free from degeneration. Finally, there were two cases in which both eyes developed xerophthalmia, but only one nerve of each animal was found to be degenerated. In eleven rabbits receiving vitamin A, carotene or both, no xerophthalmia developed. No degeneration of any kind was observed in the ophthalmic divisions of the Vth nerves in ten of these animals, but in the remaining case there were one or two fibres which showed annular degeneration (see page 22).

It would seem from the above that, although there was not complete agreement between the xerophthalmia and the nerve degeneration, on the whole it was a close relationship. It may be added that it is by no means certain that all the fibres in a nerve of this kind have been examined after treatment by Marchi's method (using frozen sections). The minute size and the fragility of the nerve tissues always make it possible that some fibres are lost, and these might be degenerate.

Two points of interest in the above summary are worthy of note:

(1) Whereas one eye may show early xerophthalmia, no macroscopical or histological evidence of abnormality may be present in the other, and in such cases the corresponding trigeminal nerve usually shows degeneration on the side supplying the affected eye only.

(2) In one case, however, degeneration was found in both trigeminal nerves, even though xerophthalmia had only been observed in one eye, suggesting that the corneal changes may be secondary to the nerve degeneration. Support for this suggestion comes from the fact that, as a

general rule, the greater the amount of degeneration in the nerve the more intense the corneal change.

Table 1 gives a picture of the histological changes in the cornea and corresponding first branch of the trigeminal nerve of rabbits at various times after the onset of xerophthalmia.

In other experiments 50 gms. daily of cabbage were added to the diets of rabbits after mild xerophthalmia had developed in both eyes. The cabbage was given to

TABLE 1

No. of days after first appearance of xerophthalmia	*Condition of cornea*	*Condition of trigeminal nerve*
1	Slight hyperplasia of epithelium	Few degenerated and swollen fibres.
6	Early keratinisation of epithelium and flattening of epithelial cells	Rather more degeneration than after 1 day.
21	Thick keratinous epithelial layer	Many degenerated and swollen fibres, including some showing typical Wallerian degeneration.

one animal, A, 10 days after the condition was first noticed. The left eye was completely clear again in 6 days and the right in 9 days; the animal was killed on the tenth day. Another rabbit, B, was not given the cabbage until 16 days after the onset of xerophthalmia. The right eye cleared in 3 days, the left in 5, and the animal was killed after 17 days of the curative therapy. From analogy with other rabbits with similar degrees of xerophthalmia, the

trigeminal nerves of these animals would be expected to show some annular degeneration, but in neither rabbit A nor B did the nerve show any obvious abnormality. In other words, healing of the cornea in both animals and in other similar cases appeared to be accompanied by recovery of the corresponding nerve.

In young *dogs* the earliest signs of abnormality vary. Sometimes changes are first seen in the cornea, where a slight opacity of part, or more often of the whole, surface occurs, and sometimes the eyelids are first affected. The cornea is not necessarily rough and dry as is common in the rabbit. The corneal epithelium may or may not be hyperplastic. It may be only one cell thick with flattened and keratinising cells overlying it. In the dog the substantia propria of the cornea is soon invaded by micro-organisms leading to ulceration of the cornea and hypopyon. The ophthalmic division of the trigeminal nerve shows degenerative changes in experimental xerophthalmia in dogs.

Young *rats* fed on vitamin A-deficient basal diets of different kinds develop eye lesions, but at varying times after the experimental diet is begun. The earliest sign is usually in the eyelids, which sometimes lose their hairs and become puffy. Corneal changes are only seen later and indeed, if the vitamin A deficiency is great, the animals may die before any obvious macroscopic corneal changes are evident. When the eyelids are swollen, without obvious xerophthalmia, the trigeminal nerve, in the author's experience, shows typical changes in the myelin sheaths. Since the trigeminal nerve supplies the eylids as well as the cornea, it is probable that this nerve also influences the maintenance of the normal structure and function of the epithelial cells in this region. The corneal covering cells, however, seem to be generally more resistant than the glandular cells of the eyelids and related tissues (Mel-

lanby, 1933). *Changes in the trigeminal nerve were also* noted in A-deficient rats by Wolbach and Bessey (1941).

When *chickens* are reared on vitamin A-deficient diets they regularly develop corneal and eyelid lesions. In the few severe cases in which nerves have been examined, the results suggest that the relationship found in the rabbit dog and rat holds good also in the chicken.

Thus, when xerophthalmia is present, even in the very early stages, in any species tested, the corresponding trigeminal nerve usually shows degenerative changes in the myelin sheaths, and in the rabbit, which has been subject to more detailed examination than other animals, their development is generally synchronous. Again, in early and slight xerophthalmia, when the corneal epithelium returns to normal as the result of adding carotene or vitamin A to the diet, the nerve also appears to return to normal. In such cases the myelin degeneration is, on the basis of other experiments, presumably of the annular type (see p. 22; also Plate I, f), which seems capable of rapid recovery. In long established cases of xerophthalmia, when the degeneration is severe and mainly of the Wallerian type, this complete return to normal of the sensory nerve has not been seen. For instance, in a dog the eyes of which had developed xerophthalmia, become infected and finally burst, the addition of vitamin A to the diet for 13 weeks *did not restore the ophthalmic division of the trigeminal* nerve to normal. At death the nerve showed, by osmic acid methods, a great many ovoids of degenerated myelin but no annular degeneration and by the Weigert technique a considerable loss of fibres.

These experimental results bring to the fore again the long-debated question of neurotrophic influence. Are the epithelial changes in the cornea in any way related to an abnormality of the sensory nerve? Is the susceptibility to infection of an eye in this condition due to the loss of

neurotrophic control? Or does the infection result from the loss of the sensory reflex mechanism to the eyelids and the reduction in or absence of blinking normally called forth by the presence of a foreign body? Is there any relationship between xerophthalmia due to vitamin A deficiency and keratitis neuroparalytica in man as seen, for instance, after removal of the Gasserian ganglion?

In *keratitis neuroparalytica* the corneal epithelium is affected in the earliest stage and assumes the same dry appearance as in xerophthalmia, but, whereas in the latter spread of the change over the whole epithelium is usually slow, in the former it is often extremely rapid. It may be that one explanation of this difference lies in the complete loss of sensory innervation in keratitis neuroparalytica, whereas the loss is often only partial in xerophthalmia. In vitamin A-deficient animals some bundles of fibres in the trigeminal nerve may be affected, the rest remaining normal. Some of the difference may also possibly be explained by the complete loss of blinking reflex after removal of the Gasserian ganglion, whereas this reflex is not entirely lost in the experimental animals referred to above. It is, however, of interest to note that clinicians have long known that in xerophthalmia the eye is relatively insensitive, a condition which itself indicates an abnormality in the sensory nerve; this is usually considered to be due to changes in the nerve-endings which occur in the corneal epithelium. The experimental results just described establish the fact that the nerve-ending changes are indicative of degeneration of the neurone.

The other well-known human disease in which both the cornea and the trigeminal nerve are often implicated is *herpes zoster ophthalmicus*. In this disease the changes in the cornea differ somewhat from those seen in xerophthalmia and keratitis neuroparalytica. Instead of the dry appearance, vesicles often form and may rupture on the sur-

face, leaving superficial ulcers which either remain discrete or become confluent, according to the severity of the attack. These ulcers do not usually perforate, but the cornea may be left permanently opaque. In xerophthalmia, on the contrary, rupture of the cornea readily occurs, especially in the dog, if the condition is not treated, but no opacity is left if the case is treated in reasonable time by the addition of vitamin A or carotene to the diet. There are obviously some important differences in the aetiological factors involved in the two conditions, which may possibly be explained simply by the presence of the herpes zoster virus. At least it is clear that in both conditions some lesion associated with the sensory nerve supply to the cornea is involved.

(b) *Night-blindness*

Evidence has been given to support the view that the pathological changes of xerophthalmia produced by vitamin A deficiency, or some of them, may well be related to loss of function of the sensory nerve supplying the cornea and related tissues, i.e. of the first division of the trigeminal nerve. Xerophthalmia, however, is only one of two main changes suffered by the eye in A deficiency, the other being loss of dark adaptation and night-blindness; and it would clearly be interesting if it were found that the optic nerve was liable to suffer degenerative changes in these conditions. In this way it might be possible to find a lesion, namely, degenerated afferent nerves common to all the derangements that develop in the eye in A deficiency.

Until recently it was thought that *night-blindness* was a frequent and easily-produced condition resulting from A deficiency, but as the result of investigations in England during the war, (see note 2, p. 59) it would not appear that deficiency of vitamin A is a common cause of night-blindness among adults in Britain and probably even less so in

the U.S.A. In children the story may not be the same. The question arises as to whether night-blindness is due to a defect of or a degenerative change in the optic nerve and retina, or whether it is primarily due to a chemical abnormality in the latter, such as deficiency or loss of visual purple. It may, of course, be a double mechanism, for instance a deficiency of visual purple in association with a nerve defect (see note 3, p. 59).

It will be clear from the account given in Chapter II that degenerative changes occur in the optic nerves of young growing animals as the result of vitamin A deficiency and that, when the vitamin is withheld for a long time, the whole of the optic nerve may ultimately degenerate and the animal may become totally blind (see p. 37). Severe changes are also produced in the retinas of young −A animals. For instance, the ganglion cells degenerate and may disappear altogether. Other changes can be seen in Plate III, c-d, which represents photomicrographs of the retinas of two rabbits, one on a vitamin A and carotene-deficient diet and the other on a diet containing carotene for periods of about 4 months. The retina of the rabbit which had no carotene or vitamin A is thinner than that of the control. There are fewer cells and less intensively staining nuclei in the ganglion and bipolar (inner nuclear) layers. Other changes are also found which cannot, however, be seen in the low power photomicrographs of Plate III, d; they include the eccentricity of the nuclei and the powdery Nissl granules in the ganglion cells. More recent work on A-deficiency in growing animals has further emphasised the ease with which pathological changes can be induced in the retina. The systematic examination of the retina in these animals by ophthalmoscope often reveals such abnormalities as *changes in the colour of the tapetum*, pallor of the disc, and papilloedema.

The eyes of adult animals are much more resistant to A

deficiency. In the experiments made on fully grown animals, only a few scattered degenerated fibres in the optic nerve have been seen, and it is not possible to say whether they have any significance.

Although both the optic nerve and the retina degenerate in vitamin A deficiency, this does not mean that there is yet proof that night-blindness is primarily due to these nerve changes. It certainly takes a long time to produce the optic nerve and retinal lesions in adult animals and, as shown in note 2 below, it is a matter of some difficulty to produce night-blindness in human adults by withholding vitamin A from the diet. On the other hand, it is easy to produce optic nerve and retinal lesions in growing animals by this means. It is not known, however, whether under these conditions night-blindness is readily produced either in young animals or children. If it were so, the facts would in general support the view that night-blindness and optic nerve and retinal degeneration, due to vitamin A deficiency, were related.

Summing up this part of the investigation made with the object of gaining some insight into the mode of action of vitamin A on the eye, it can be said that the general outcome was regarded as supporting the view that an afferent nerve disorder was a common factor in both pathological conditions induced in this organ by vitamin A deficiency. The condition of xerophthalmia, on the evidence obtained, was usually associated with a lesion of the first division of the trigeminal nerve, the sensory nerve supplying the cornea and surrounding orbital tissues. The rôle of the optic nerve and the retinal cells in the development of night-blindness could not be easily excluded when it was seen how susceptible, in young animals, these tissues were to degenerative changes in A deficiency. The general result has indeed been to suggest the

importance of the nerves of the eye in relation to the abnormalities which develop when the vitamin is lacking. Not only are these abnormalities usually accompanied by degeneration of the nerves concerned, but when, as in the adult, it is difficult to produce the nerve degeneration, it is also difficult to produce the specific indication of A deficiency such as xerophthalmia or night-blindness. These eye investigations helped to drive home the newly described and apparently unreasonable fact, namely, that vitamin A deficiency was closely bound up with afferent nerve degeneration. On the other hand, it cannot be claimed that any insight was gained by this particular investigation which would explain how these widespread degenerative changes were produced in the afferent nerves of the orbit when vitamin A was absent from the body.

The above account of the degenerative changes of the afferent nerves of the eye in relation, respectively, to xerophthalmia and night-blindness, has been written along the lines and under the same limitations as would have described an investigation written up in 1934, when the work was actually done. Since that time, there have been some developments which, although they do not solve the particular problems at issue, have an important bearing on them. These facts are set out in the following notes:

Note 1: Epithelial hyperplasia and sensory nerve degeneration

In the years that have elapsed since the discovery of the frequency with which the ophthalmic division of the Vth nerve shows degenerative changes in A deficiency, no good evidence has been forthcoming to support the proposition that hyperplasia, metaplasia and keratinisation of the corneal epithelium, or indeed of any epithelium in A deficiency, is directly due to the sensory nerve degenerative change. King (1936) attempted in the author's laboratory to obtain evidence on this point in the case of the gingival epithelium of dogs and rabbits, which M. Mellanby (1930) had shown to be liable to hyperplastic and other changes in vitamin A

deficiency. M. Mellanby and King (1934) had also shown that the sensory nerves of teeth and jaws degenerated in A deficiency and, therefore, it was possible to test in this case whether the loss of sensory innervation affected the growth of the dental epithelium For this purpose, King removed part of the inferior dental nerve on one side in rabbits and dogs. No difference, however, could be found in the periodontal tissues on the operated and non-operated sides. It is true, as pointed out by King, that the sensory loss in these animals on the operated side was not complete since the lingual and long buccal nerves were intact. However, these experiments showed that interference with some of the sensory nerve supply leaves the innervated epithelium unchanged The experiments failed to establish any direct relationship between the sensory nerve degeneration and the tendency to epithelial hyperplasia.

Note 2: Night blindness and vitamin A deficiency in adults

A wartime investigation into the development of night blindness was made on a group of human volunteers, 16 of whom consumed a *vitamin A-free and low carotene diet for periods varying from* 10½ to 25 months (Medical Research Council Special Report Series No 264). Only in 3 individuals, after 9, 10 and 20 months respectively, did signs of night blindness, as measured by the Wald adaptometer, develop Livingston's rod scotometry method was applied from the tenth month of the experiment, and the results suggest that early changes might be present in some other subjects Five of the volunteers remained on the vitamin A-free diet for 18-25 months and in four of these no loss of dark adaptation was recognised In none of the 16 subjects were changes observed by examination with the slit lamp, but eye discomfort variously described as soreness, streaming, pricking, or feeling of sand in the eyes, occurred in 11 of the deprived group and in only one of those not deprived.

Note 3: Night blindness—a direct chemical relationship between vitamin A and night blindness

Since this work on degeneration of the optic nerve and retina was published (Mellanby 1933), the importance of a direct chemical relationship between vitamin A and night blindness without the intervention of the retinal nerve mechanism has become greatly strengthened by the work of Wald (1935). He showed that visual purple behaves as a conjugated protein in which retinene is the prosthetic group and that the visual processes involve a chemical

cycle in which vitamin A is a precursor, as well as a product of decomposition. According to Wald, visual purple is synthesised in the retina by two processes, (1) the reversion from visual yellow (retinene) and (2) regeneration from colourless substances, among them vitamin A. The regeneration of visual purple from yellow retinene appears to be a simple reversal of photolysis. The synthesis from vitamin A, however, occurs only in an eye in which the relation of the retina to the pigment epithelium has remained undisturbed. These views have been strengthened by the work of Hawkins and Hunter (1944) and by Morton and his colleagues (1944-1946), who found that retinene was vitamin A aldehyde. Similarly, in the retina of fresh water fish Wald (1937) whilst studying porphyropsin, the visual purple analogue of vitamin A_2 (Edisbury, Morton & Simpkins 1937) discovered retinene$_2$ since shown by Morton et al. (1946) to be vitamin A_2 aldehyde.

It is clear that these recent chemical discoveries, linking vitamin A with the visual purple and the cycle of chemical changes it undergoes in light and dark adapted eyes, leave less room than ever for an intermediary nervous mechanism, although the need for such for the conversion of vitamin A to visual purple may be indicated by the fact that this change seems to require an intact retina closely associated with the pigment epithelium. At the same time, however, recent work on the relation of vitamin A to the structure and function of the optic nerve and retina has emphasised still further how susceptible these are to vitamin A deficiency, especially in young animals.

We seem forced at the present time, therefore, to take the view that, far from unifying the problem of vitamin A in relation to the eye and especially to retinal function, the general trend of modern research is to make the matter more complicated. The evidence supports the view that vitamin A has two distinct actions with regard to vision: (1) It is an essential element in the synthesis of visual purple and thus is of direct chemical importance to vision in lights of low intensity, and (2) It is essential in young animals for the maintenance of the normal structure of the optic nerve and retina. But in spite of the apparent independence of these actions, it may well be that night-

blindness in vitamin A deficiency is related to optic nerve and retinal function.

2. Neurotoxins in Cereals

Brief reference will now be made to the experimental work done to test the hypothesis that the nerve degeneration described in Chapter II was due to a toxic substance in cereals, the action of which was normally antagonised by vitamin A (Mellanby, 1926). The hypothesis was parallel to that of rickets put forward some time earlier (Mellanby, 1922), namely, that cereals contained varying quantities of an anticalcifying or rachitogenic substance whose action was normally prevented by vitamin D. As will be seen later (Part II) the hypothesis in the case of calcification proved to be true. On the other hand, the presence of a neurotoxin in cereals antagonised by vitamin A was not established and indeed, as will be seen in Chapter VII, the primary abnormality was not in the nervous system. There is reason to believe, however, that a parallel hypothesis will be found to explain the aetiology of some of the nerve degenerative diseases of nutritional origin which affect human beings, and which, by their similarity in nerve pathology, seemed at the time to be closely related to the experimentally produced nerve lesions described in Chapter II. In 1926 when the idea of a neurotoxin in cereals was suggested one of the experimental facts which appeared to support it was that, when cereals were removed from an A-deficient diet and replaced by potato, incoördination and degenerative changes in nerves developed much more slowly and were less severe. At the time these experiments were made it was not known that carotene had the same action as vitamin A, although further consideration at a later date suggested that the carotene in potato was probably part of the explanation.

Differences in the carotene content of various cereals were, on other occasions also, disturbing factors, and it may be of interest to note that, whereas yellow corn (maize) with its relatively large carotene content entirely or largely prevented the nerve degeneration produced by an otherwise A-deficient diet, white corn (maize) allowed severe degeneration to develop.

Other facts, including the relative effect of different cereals and parts of cereals in producing nerve degeneration when associated with an A-deficient diet, supported the hypothesis of a cereal neurotoxin. In particular, the addition of the germ or embryo of a cereal, especially that of rye, to an A-deficient diet increased the nerve degeneration. *This suggested that convulsive ergotism in man might* be due to a vitamin A deficiency associated with a neurotoxin in ergotised rye. Many experiments were made on animals to see whether this disease could be regarded as due to such a neurotoxin in ergot, the action of which was determined by vitamin A or other substances. From animal experiments with crude ergot this appeared probable (E. Mellanby, 1931), but all efforts to isolate a neurotoxin which might be regarded as responsible for the development of convulsive ergotism, failed.

Lathyrism is another human disease with nerve lesions which it was thought might be caused by a neurotoxin and which was studied in the laboratory. The lesions of the nervous system produced in animals whose diets consisted largely of lathyrus peas were, however, different from those experienced by human beings and again no success was obtained in demonstrating the presence of a neurotoxic agent, although some suggestive evidence was obtained with the variety of pea called Akta. Lathyrism is a major nutritional problem in some parts of India, and convulsive ergotism has in the past caused enormous destruction of human life in Russia, Germany and other European

countries. It is interesting to note that Frederick the Great is said to have got rid of the disease in Germany by making people grow and eat potatoes. Lathyrism and convulsive ergotism only appear in semi-starving populations when the diet is very limited in quality and quantity. Another disease in which fibres of the central nervous system are affected is subacute combined degeneration of the cord associated with pernicious anaemia.

Although it proved impossible at the time to establish either that the degenerative changes in the cord of the experimental animals were due to a neurotoxin in cereals which was normally antagonised in its action by vitamin A, or that the experimental results shed light on degenerative diseases of the nervous system of nutritional origin in man such as pellagra, convulsive ergotism and lathyrism, it still seemed likely that these latter diseases would ultimately prove to be caused by substances of this nature.

In an article published in 1937 (Mellanby) the following remarks were made: "In all these conditions (pellagra, convulsive ergotism and lathyrism), there is reason to believe that toxic agents associated with maize, ergotised rye, and certain lathyrus peas respectively, are playing a part in the degeneration of the nervous system. There is also good evidence that these toxic effects do not occur when the diet is rich in protective foods, especially those containing vitamin A and probably other vitamins, such as those of the vitamin B complex."

In the light of this statement it is of interest to note that in the case of pellagra recent discoveries have proved that specific chemical and nutritional factors, harmful and protective respectively play a part in its aetiology. Thus Krehl, Tepley, Sarma and Elvehjem (1945) showed that corn contains a substance which exerts a growth-inhibiting action on rats receiving a ration low in tryptophane and that nicotinic acid (the PP or pellagra-preventing vita-

min) causes restoration of growth. Woolley (1945) then found that 3-acetyl-pyridine, an analogue of nicotinic acid, antagonised the action of nicotinic acid and of nicotinamide and produced in mice a pellagra-like syndrome. He next showed that tryptophane also antagonised the toxicity of 3-acetyl-pyridine and prevented the pellagra-like manifestations called forth by the ketone in mice. On the basis of these results, Woolley postulated that the pellagragenic action of corn is due to a specific chemical substance, possibly an analogue of nicotinic acid, which acts by preventing the action of nicotinic acid and nicotinamide by competing with them. There seems but little doubt that corn contains a pellagragenic substance, the action of which is antagonised by a vitamin and in this case also by an amino acid; the chemical constitution of this toxic substance in corn will no doubt soon be discovered. The vitamin conditioning the toxic action is not vitamin A as was thought possible when the present experimental work was done (Mellanby, 1934a, 1937b), but a member of the B complex.

In passing it may be of interest to note that here is another instance of the principle of biological competition, a principle which has had such great success in explaining biochemical problems in recent years. The idea was first formulated by Quastel and Wooldridge in 1928, when they found that malonic acid inhibited the action of succinoxidase. The principle was extended by Fildes (1940) and Wood (1940) to explain the action of sulphanilamide, which they found competed with para-amino benzoic acid, a metabolite essential for cellular existence. The example given above of the toxic action of 3-acetyl-pyridine which is antagonised by nicotinamide and tryptophane is another instance of biological competition and it is reasonably certain that the action of the pellagragenic substance in corn will prove to be of the same type. Clearly such bio-

chemical actions are ideal for allowing conditional toxic action of the kind now under discussion. Substances having a toxic action which are antagonised by a vitamin have been called by the present writer 'toxamins' (1926, 1931a, 1937b). The first of these to be demonstrated was phytic acid, the anticalcifying substance in cereals. It will be seen in Part II how its action is antagonised by vitamin D and how, although the mode of interaction of vitamin D and phytic acid is still unknown, a part of the chemical mechanism is probably competition between phytic acid and phosphoric acid for calcium in the alimentary canal and the consequent interference with the absorption of a calcium phosphate compound under the stimulus of vitamin D.

Although investigators have not yet included in their biochemical and clinical studies the nervous aspect of the pellagra syndrome from this new angle, recent observations on subacute combined degeneration of the cord, a condition associated with pernicious anaemia, indicate that central nerve degeneration of nutritional origin will ultimately fall into line with the other syndromes under discussion. The renewed scientific interest in subacute combined degeneration is due to the discovery that folic acid therapy cures the blood picture of pernicious anaemia, but does not prevent the development of cord degenerative changes (Spies and Stone, 1947). Extract of liver, however, both cures the blood picture and prevents the cord degeneration. Whatever the ultimate mechanism may prove to be, it is clear that one factor determining the development of cord degeneration is the absence of a nutritional substance found in liver, whose identity is at present unknown or at least unrecognised.

The researches described in this chapter have certainly had some interesting developments, but taking all the investigations together, it must be confessed that the

search for a neurotoxin in cereals was not at the time successful, and the results threw but little light on the process of vitamin A activity. As knowledge of the lesions in the central and peripheral nervous systems produced by diets deficient in vitamin A accumulated, it became more and more difficult to accept the hypothesis that the nerve degeneration was *directly* determined by a toxic action conditioned by the absence of vitamin A. Such a hypothesis assumed that a special chemical or enzymic reaction was necessary for the maintenance of structure and function of certain groups of nerve cells which seemed to bear no particular relation to one another, while other groups were independent of this reaction. For instance, what possible metabolic factor could account for:

(1) the large amount of degeneration between the pons at the level of the entrance of the Vth cranial nerve and the lower cervical region of the cord and the usually normal appearance of the higher parts of the brain: (an animal might be deaf and blind and completely incoördinate in movement and yet the cerebral cortex and the brain above the red nucleus would often be normal in appearance);

(2) extensive degeneration, not only of the primary ascending neurones but also of some of the secondary ascending neurones, e.g. the cerebellar tracts and the fillet;

(3) the limitation of the degeneration of the descending fibres to those starting in and below the mid-brain;

(4) the strikingly uneven distribution of degeneration which sometimes occurred on the two sides of the body, especially in the fibres having their origin in the dorsal spinal roots?

Now that answers to some of these questions have been obtained, it is no longer easy to enter into the perplexity of that period. It may, however, be of interest to those about to start a career of research to know that for several years the author, in spite of great and continuous effort, re-

mained completely ignorant of some of the main factors concerned in these problems. Although such factors are so obvious, when once seen, that their escape from detection is almost incredible, the fact is that they were also missed by all who studied the biological action of vitamin A.

Chapter IV

A NEW LINE OF ATTACK

A time came when the unsatisfactory explanation, which could be neither proved nor disproved, of the events leading up to nerve degeneration in animals fed on vitamin A-deficient diets impelled a return to the problem; but instead of continuing from where the investigation had left off, it was decided to begin de novo.

It was still felt that the incoördination of the −A animals was the sign to be studied first and indeed that the vestibule and its nervous connections might well hold the main secret. It will be remembered that, in the early days of the work, the VIIIth nerve and its divisions were the first to be examined for degeneration, using Marchi and other histological techniques. The degenerative changes found earlier both in the cochlear and the vestibular divisions, although providing an explanation of deafness and incoördination in the −A animals did not fulfil the hopes that had been raised of further progress. In the new study it was thought desirable to delve deeper and to examine the cochlea and labyrinth to see the condition not only of the nerves but also of their cells of origin in −A as compared with +A animals. Tissues of dogs, rabbits and rats were fixed by intra-arterial injection of Wittmaack's solution (1926) and parts of the temporal bones containing the labyrinthine capsules were removed, decalcified and embedded in celloidin; serial sections were then cut and stained by various methods (*E. Mellanby, 1938*).

Examination of the sections suggested an immediate explanation of the main problem, for it was seen that in the *labyrinth of the −A animals there were masses of* newly formed bone both in the modiolus and in the internal



PLATE V

Photomicrographs (× 14) of sections of cochleas and surrounding bone of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Note: Great narrowing of internal auditory meatus and compression of nerve by bone over-growth (NB) in −A animal (b).

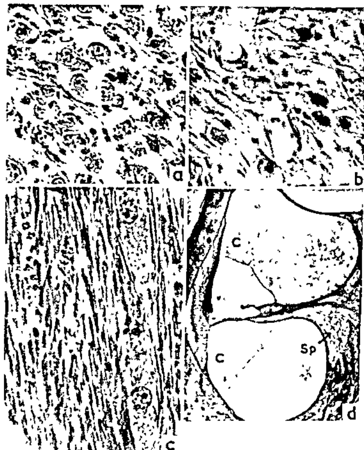


PLATE VI

(a) and (b) Photomicrographs (× 325) of cells of the spiral ganglia of two litter-mate dogs of the same age.

(a) +A diet

(b) −A diet.

Note: Definite Nissl granules and well-defined nuclei in (a), whilst in (b) nearly all the ganglion cells have completely degenerated and the few remaining are shrunken and the protoplasm is homogeneous. Cytoplasm is devoid of granules in (b).

(c) *Photomicrograph (× 325) of cells of Scarpa's ganglion of a −A dog*

Note: Although these cells are squeezed and elongated, the internal structure has remained apparently normal.

(d) Photomicrograph (× 33) of the basal whorl of the cochlea of a −A dog.

Note: The cells of the spiral ganglion (Sp) have disappeared, together with the peripheral nerve fibres to the organ of Corti. Albuminoid coagula (C) are obvious in both the scala tympani and scala vestibuli, indicating a condition of serous labyrinthitis. The organ of Corti is very abnormal.

auditory meatus, which clearly interfered with and, in long-established cases, ultimately destroyed both the cochlear and vestibular divisions of the VIIIth nerve (Plate V a & b). The amount of bone overgrowth seemed to determine the degree of incoördination and the abnormal behaviour of the dogs. The new bone was periosteal in origin: that in the labyrinth, especially the part adjacent to the brain, became abnormally thick, so that the helix and vestibule were more deeply placed in the labyrinth, causing an increase in length of the VIIIth nerve. In severe cases, the internal auditory meatus appeared to be completely blocked by the bone overgrowth, but further examination of serial sections usually revealed that the nerve, although twisted and further lengthened, still had a passage through the bone from the labyrinth into the brain stem. The cells of the spiral ganglion of the cochlear nerve degenerated relatively early and in severe cases all disappeared (Plate VI a & b). On the other hand the cells of Scarpa's ganglion, the origin of the vestibular division, were much more resistant to pressure; they could be squeezed into abnormal shapes (Plate VI c), and yet appeared to retain their minute structure and probably their function. However, even in these cells, there was a limit to their resistance and many, in long-continued experiments, were found to be completely destroyed.

The relative stability of the vestibular compared with the cochlear division of the VIIIth nerve to mechanical pressure and injury observed in these experimental animals is an established fact in man, well known to otologists. It must be remembered that the cochlear nerve resembles the optic nerve in that injury to any part of the neurone destroys it entirely. The vestibular nerve, however, reacts like the majority of nerves and follows the Wallerian law of degeneration, i.e. the nerve degenerates from the point of injury peripherally.

It was further found that, in addition to the bone overgrowth and nerve degeneration, the cochleas of severely affected −A animals developed a serous labyrinthitis with albuminoid coagulation of the perilymph (Plate VI d). This condition had probably no relation to the VIIIth nerve destruction but, as will be seen later, may have been determined by changes in the cerebro-spinal fluid of the subarachnoid space with which the perilymph communicates by means of the cochlear duct (E. Mellanby, 1938). It is likely that the labyrinthitis destroyed the organ of Corti in the experimental dogs, as it is known to do in man.

The following experiment supports this view. Two dogs, X and Y (aged 8 weeks) of the same litter, were placed on a vitamin A-deficient diet and after 15 weeks were badly affected. One of the dogs, X, was then given 30,000 I.U's of vitamin A daily for 27 weeks, the second, Y, remaining on the A-deficient diet. The experimental feeding period thus ended after 42 weeks. Sections of the labyrinths showed that in both X and Y the cochlear divisions of the VIIIth nerve had been destroyed, but whereas Y had labyrinthitis and a degenerated organ of Corti, X had no labyrinthitis and the organ of Corti was nearly normal. It seemed as if the addition of vitamin A to the diet of X had cleared up or checked the onset of labyrinthitis and thereby prevented the degeneration of the organ of Corti but it had been added too late to prevent the degeneration of the cochlear nerve. In Y, on the other hand, not only had the nerve degenerated but the labyrinthitis had allowed or been responsible for the degeneration of the organ of Corti.

The outcome of this new investigation, therefore, was the discovery that a deficiency of vitamin A in the diet brought about the development of excessive newly formed periosteal bone and that the alteration in shape of the bone was responsible for the degeneration of the VIIIth nerve, of which the cochlear neurones were most affected. All the

evidence supported the deduction that the bone overgrowth was directly responsible for the degeneration and, from the point of view of the whole investigation, this deduction was of fundamental importance. Could abnormal bone growth in A deficiency be also responsible, for instance, for degeneration of the optic nerve, the trigeminal nerve and the dorsal root ganglion? Could it be responsible, not only for the degeneration of the first ascending neurones having their origin in the dorsal root ganglion, but also for the degeneration of endogenous tracts in the cord, such as the dorsal and ventral spino-cerebellar tracts, seen in the earlier investigations to be present in A deficiency? The task of examining bone growth in −A animals and relating it to the condition of the nervous system seemed a formidable one, but an attempt was made along these lines and some of the results obtained will now be described.

Chapter V

BONE DYSPLASIA AND THE CENTRAL NERVOUS SYSTEM

An account will now be given of those effects of vitamin A deficiency on the bones surrounding the central nervous system and the resulting changes in that system that can be seen or recorded macroscopically. Both the brain and the spinal cord are directly affected by the bone changes. The present account deals almost entirely with results obtained on dogs, but a brief reference will be made to results obtained in other young animals, which will suffice to show that, although there may be species differences in details of reaction to vitamin A deficiency, the general outcome is the same independently of species.

1. The Brain (Dog)

The first examination to be made was that of the skull bones. It was soon obvious that these showed gross deformity in −A animals and that the malformed bones were greatly affecting the disposition of the brain.

(*a*) *Shape*

Diagrams of mesial sagittal sections (Fig. 20) of the skulls of two litter-mate puppies which had grown at the same rate during the experimental period show some of the regions that are specially subject to bone overgrowth in A deficiency. It will be seen that the brain of the −A animal is more tightly packed into the cranial cavity than is that of its +A litter-mate. This is a common finding. The bones showing the greatest overgrowth are those of the posterior fossa, i.e. those surrounding the cerebellum, medulla oblongata and the pons. The importance of this fact is discussed later (see p. 151).

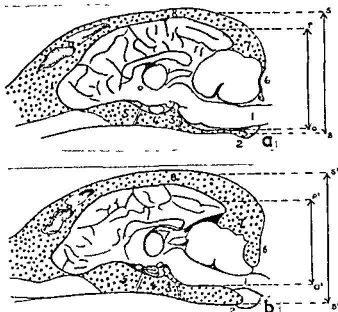


Fig. 20. Drawings of mesial sagittal sections of skulls of two litter-mate dogs of the same age.

(a) +A diet.

(b) −A diet.

Note: (1) Great increase in thickness of bones surrounding the posterior fossa and, to a less extent, other bones in (b) as compared with (a).

(2) Compression of medulla and cerebellum and pushing back of the posterior part of the cerebellum into the foramen magnum in (b)

(3) Differences in measurements PO, P'O', and similarity of SB, S'B', showing lack of absorption of bone on internal surface in (b)

(4) The calcified tentorium cerebelli (marked black) in the −A animal (b)

Key to drawings: 1. Foramen magnum. 2 Basi-occipital. 3. Posterior clinoid process 4 Basi-sphenoid 5. Anterior clinoid process 6 Supra-occipital. 7. Occiput. 8. Parietal 9 Frontal.

In particular, all parts of the occipital bone, both above and below the brain stem, are greatly enlarged in Fig. 20b as compared with the normal. Passing forward from the

supra-occipital the parietal bone is also much thickened in its posterior part, but the thickening is reduced as the frontal bone is approached. Similarly, at the base of the skull, passing forward from the hypertrophied basi-occipital bone, the enlargement of the basal portion of the sphenoid, although definite, is not as great as that of the occipital bone.

The effect of this enlarged bone at the posterior end of the skull is to cause increased pressure on the cerebellum and medulla oblongata and a consequent alteration in their shape. The cerebellum in Fig. 20b can be seen to be flattened on its dorsal surface by the thickened supra-occipital bone. In the particular skull illustrated the tentorium cerebelli has become calcified and a wedge of bone separates the occipital lobe of the cerebrum from the anterior dorsal surface of the cerebellum, a condition only found in relatively advanced stages of A deficiency. It is also seen that the posterior portion of the cerebellum is pressed backwards through the foramen magnum between the occipital bone and the dorsal surface of the medulla oblongata, a space which is normally part of the cisterna magna. This intrusion of part of the cerebellum into the cisterna magna can be better appreciated in Plate VIIb (compared with Plate VIIa, cisterna magna of a normal dog).

The medulla, instead of being cylindrical in that part just ventral to the cerebellum, as in Fig. 20a (+A animal), is compressed in Fig. 20b (−A) and the 4th ventricle and the aqueduct of Sylvius are narrowed and reduced in capacity. The overgrowth of the occipital bone surrounding the foramen magnum lessens the area of the aperture through which the posterior end of the medulla passes and presses on this part of the nervous system. Actual narrowing of the dorsiventral cross-section of the medulla at this point can be seen by comparing the two skulls. In the former there is also a change in the posterior clinoid process at the

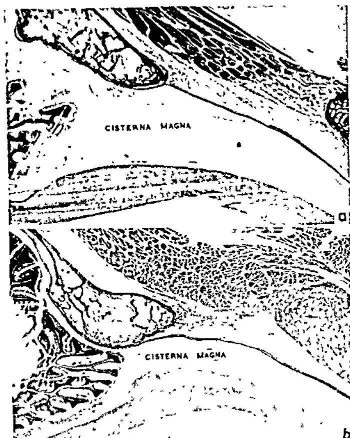


PLATE VII

Photomicrographs (× 4) of mesial sections through the foramen magnum of two litter-mate dogs of the same age
(a) +A diet.
(b) −A diet

Note: Intrusion of the cerebellum into, and reduced capacity of, cisterna magna in −A dog (b).

posterior end of the sella turcica. It is enlarged dorsally and bent forward at its free end. In some −A dogs this overgrowth has been so great that it has compressed the pituitary body (see p. 85).

While these are some of the more obvious defects seen in the sagittal sections of −A animals, removal of the brain reveals other abnormalities. As those around the foramina of the cranial nerves will be described in detail later (p. 96), they will only be referred to here in terms of naked eye changes. Most prominent is the hypertrophy of the petrous portion of the temporal bone, which is swollen and loses its fine outline. It is now a bulbous mass of cancellous bone with a very thin covering of compact tissue. The foramen of the VIIIth nerve is crenated and reduced in size, as is also the fossa of the paraflocculus; both of these apertures may, in severely affected animals, be nearly occluded but they have never been found to be quite closed. All this distorted growth must further reduce the space available for that part of the brain contained in the posterior fossa and thereby increase the pressure on the pons, medulla oblongata, cerebellum and the nerves closely related to them.

Passing forward to the part of the brain within the middle cranial fossa, the direct pressure of the bone is less than in the posterior fossa, since neither the sphenoid nor the parietal shows the great thickening noted in the temporal and occipital bones.

The bone changes in the neighbourhood of the anterior cranial fossa are also relatively small although, as will be seen later (p. 99), they may be large enough to damage the IInd nerve.

The following measurements of skulls (Table 2) illustrate some of the main overgrowths which directly affect the brain. They represent measurements of comparable regions of the skull bones of two dogs (litter-mates) on vitamin A-rich and vitamin A-deficient diets respectively. Reference to Fig. 20 shows that the outside measurements SB, S'B' of the two skulls are similar, 54 mms. in the +A as against 58 mms. in the −A animal. When, however, the

inside measurements PO, P'O' are examined, it is seen that the −A animal, the skull of which has the larger overall size, has the smaller cranial cavity, 37 mms. as against 44 mms. in the control. The increased thickness of the cranial bones in the −A animal, indicated in Table 2, offers an explanation of this fact, just as the hypertrophy of the basi- and the supra-occipital bones explains the reduced size of the foramen magnum (12.5 mm. instead of 15.8 mm., as in

TABLE 2

Bone	+A dog	−A dog
	mm.	mm.
Basi-occipital (2) (thickness)	4.2	8.5
Basi-sphenoid (4) (thickness)	3.8	6 0
Supra-occipital (6) (thickness)	3.0	6.9
Occiput (7) (thickness)	11.5	18 0
Parietal (8) (thickness)	4.0	6.0
Frontal (9) (thickness)	5.0	6.0
Posterior clinoid process (3) (dorsal-ventral length)	9.0	12.0

The figures in parentheses relate to the diagram shown in Fig 20.

the +A animal). (For further discussion of these facts see p. 143.)

(b) *Intracranial Pressure*

That the abnormal bone growth increases the pressure on some regions of the brain is undoubted, for, as shown above, the brain is actually deformed in the posterior cranial fossa. The question arises as to whether there is a general increase in intracranial pressure and, if so, whether it is equal in all parts. In this connection a number of points must be considered. In the first place, as already seen, the overgrowth is proportionately much greater in the bones

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A similar increase would be expected in the subarachnoid space surrounding the brain and cord, in the cisternae at the base of the skull and, if the foramina of Luschka are still patent, in the 4th ventricle. The mere fact that there is usually fluid in the cisterna magna probably indicates that, even in dogs fed on diets deficient in vitamin A over long periods the foramina of Luschka are open, because it is generally accepted that the cerebrospinal fluid is secreted in the choroid plexus, and that there is very little of this tissue in the cisterna magna, the little that is present protruding from the fourth ventricle through the foramina of Luschka. In the dog, the foramen of Magendie is absent (Dandy and Blackfan, 1914, 1919) and, as far as is known, there is no other outlet, although it has been suggested that there is a connection between the lateral ventricles and the cisterna interpeduncularis. Complete closure of the foramina of Luschka by pressure from abnormal bone growth might be expected, therefore, to prevent the passage of fluid from the brain ventricles to the subarachnoid space, cisternae, etc., including the cisterna magna, and any fluid present in the latter space would tend to be absorbed. In those cases, referred to above, where no evidence of cerebrospinal fluid in the cisterna magna of −A dogs could be obtained by puncture, it is possible that the foramina of Luschka were occluded: no certain evidence of complete closure of these foramina has, however, so far been obtained. On the other hand, there is no doubt that in −A as compared with +A dogs both the 4th ventricle and the foramina of Luschka may be greatly reduced in size and the choroid plexus may be more closely packed into them (see Fig. 21).

Let us now turn to the other brain ventricles, the 3rd and lateral. If the aqueduct of Sylvius were completely occluded, it is known from Dandy and Blackfan's experiments on dogs, (1914, 1919) that a condition of internal

surrounding the hinder part of the brain. If there were no obstruction between the posterior and middle cranial fossae, the increased pressure of the hypertrophied bones on the pons, cerebellum and medulla would be spread over the whole brain via the cerebrospinal fluid. This spread, however, is probably hampered on the dorsal aspect by the tentorium cerebelli; in some of the −A animals this obstruction may be more effective because, instead of being membranous as in normal animals, the tentorium is partially calcified (see Fig. 20b). The cerebrospinal fluid, however, will presumably tend to distribute the pressure via the basal cisternae and subarachnoid space, which are continuous between posterior and middle fossae. Direct observation indicates that the excess bone round the posterior fossa affects the 4th ventricle and the cisterna magna and their contents (Plate VII). The capacity of these spaces is reduced and the pressure of the cerebrospinal fluid in them might, therefore, be expected to be increased.

An attempt was made to record the pressure of the cerebrospinal fluid in the cisterna magna of some of these experimental animals. There is no difficulty in doing this in dogs whose diets contain vitamin A, but in those receiving the deficient diet the distorted bone growth makes it difficult to reach the cisterna with a needle, the reduced size of which (Fig. 20b and Plate VIIb) also increases the risk of damaging the medulla, so that it may be difficult to obtain samples uncontaminated by blood. In one or two of the animals on the A-deficient diets there appeared to be little or no cerebrospinal fluid in the cisterna magna. This point will receive consideration later (see p. 84). The pressure of the fluid in the cisternae has been successfully recorded in four −A and four +A dogs. The average pressure in the −A group was 100 mm. of water, compared with 58 mm. in the +A group. Moore and Sykes (1940) found increases in intracranial pressure in −A calves.

of the −A dogs? This can be seen in Fig. 22. A condition resembling internal hydrocephalus is present in the −A



FIG 22, a and b. Photomicrographs of coronal sections through the lateral (Lat.) and IIIrd ventricles of two litter-mate dogs of *the same age*.
(a) +A diet.
(b) −A diet.
Note: The distention of the ventricles (internal hydrocephalus) in the −A dog (b).

dog and both the lateral and 3rd ventricles are expanded. The expansion is not, however, of the same order as that produced by Dandy and Blackfan, in whose experimental

hydrocephalus would result. By analogy, it would be expected that, if the pressure due to excessive bone growth was sufficient to occlude the aqueduct of Sylvius, the cerebrospinal fluid would continue to be secreted by the choroid plexus; and since the fluid could not pass into the 4th

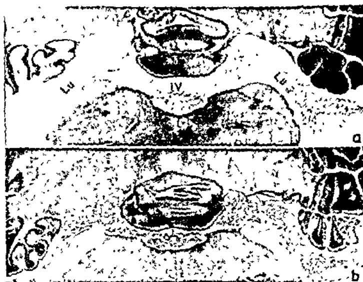


FIG. 21, a and b Photomicrographs of coronal sections through the 4th ventricles and lateral recesses (foramina of Luschka), of two litter-mate dogs of the same age.
(a) +A diet
(b) −A diet
Note: In the −A dog (b):
(1) The reduction in size of the 4th ventricle (IV), especially in the *dorso-ventral diameter;*
(2) The packed appearance of the choroid plexus;
(3) The *reduced diameter of the foramina of Luschka (Lu).*

ventricle and thence to the cisternae and the subarachnoid spaces to be absorbed, the accumulation would increase the pressure in the lateral ventricles, expand them and cause an internal hydrocephalus.

What, then, is the state of the lateral and 3rd ventricles

with that of the normal animal. It might be expected therefore that one of the known functions of this fluid, namely to distribute pressure evenly and thereby to lower it at local points of excessive pressure, would be impaired in −A animals.

(c) *Blood Supply*

Restriction of blood supply due to bone dysplasia may also be of some significance. It is undoubted that the bony channels through which the vascular supply of the nervous system is carried are often much constricted in vitamin A-deficient as compared with normal animals, but in the cases so far examined are never occluded. Whether the constriction ever interferes with the nourishment of the nervous system under these conditions is not known. In the region of the cerebellum and brain stem, however, where the pressure effects are great, some reduction of the blood supply probably occurs, but it seems unlikely that this can be responsible for the widespread and intense nerve degeneration observed in this position.

(d) *The Pituitary Body (Hypophysis)*

In severe cases of A deficiency, especially in those in which internal hydrocephalus has developed, the pituitary body may be severely compressed (Mellanby 1939 a and b). The infundibular stalk of the dog is hollow and connects the IIIrd ventricle with the infundibular cavity of the pars nervosa. Thus any increase of pressure within the IIIrd ventricle will be transmitted to the pituitary body. Increases in pressure and volume of fluid may be so great that they distend the pituitary rather like a small balloon, and tend to force it against the basi-sphenoid bone and the posterior clinoid process. The latter is one of the structures which undergoes large changes. It grows in an antero-dorsal direction and the head becomes considerably enlarged.

dogs the aqueduct of Sylvius was completely occluded by direct surgical methods. It is possible, therefore, that the internal hydrocephalus produced in the diet experiments is not due to the complete occlusion of the aqueduct, but rather to the increased pressure in the 4th ventricle and the cisterna magna being communicated to the 3rd and lateral ventricles.

This, however, is a complicated problem, for it is necessary to consider not only the actual direct pressure effects of the abnormal bone growth on different parts of the brain and cerebral fluid and the patency of the aqueduct of Sylvius and the foramina of Luschka, but also the relative rate of secretion of cerebrospinal fluid by the choroid plexus and its absorption in the subarachnoid spaces. Nevertheless the foregoing examination shows that there is often a large *increase of pressure in the cerebrospinal fluid in —A dogs* and that, associated with it, there is:

(1) Compression and reduction in size of the 4th ventricle, cisterna magna and other cisternae at the base of the brain;

(2) A condition of internal hydrocephalus with expansion of the lateral and 3rd ventricles.

Whether there is ever complete occlusion of the aqueduct of Sylvius and the foramina of Luschka, either temporarily or permanently, is not known, but the evidence is rather against it, although there is certainly definite narrowing of these passages. In the —A dogs the whole brain and probably the spinal cord are subjected to increased pressure. The cerebrospinal fluid mechanism tends to diffuse this rise of pressure to all parts of the central nervous system, but whether it succeeds in severe cases of vitamin A deficiency or whether it fails because of a break in continuity of the fluid is not known. Where the anatomical changes above described are great, the total capacity of the spaces containing cerebrospinal fluid is much reduced as compared

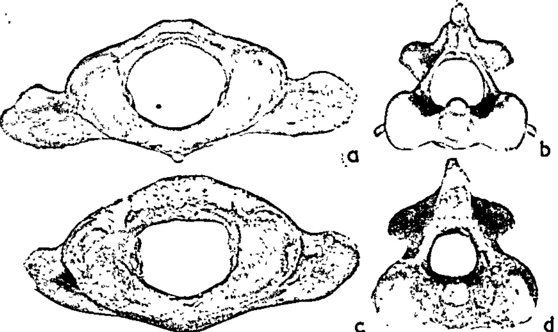


PLATE VIII

Photographs of atlas and axis vertebrae of two litter-mate dogs of the same age.
(a) and (b) +A diet.
(c) and (d) −A diet.
Note: (1) The overall sizes of the comparable vertebrae are not greatly dissimilar
(2) The vertebrae of the −A animal are coarse and blunted and have lost their delicate outline.
(3) The spinal canal in the vertebrae of the −A animal is smaller than that of the +A.

Thus the cells close to the postero-dorsal surface of the pituitary are forced by the cerebrospinal fluid against the enlarged head of the posterior clinoid process. These cells are part of the pars anterior for, in the dog, this lobe laps around the pars nervosa. Some of these cells have been seen to be elongated and arranged in rows parallel to the surface of the gland because of the pressure effect. Whether the functions of these distorted pituitary cells are affected by the pressure is not known.

2. The Spinal Cord (Dog)

A description of the changes found in the vertebral columns of vitamin A-deficient animals and the effect of such changes on the spinal cord will now be given. It may be said at once that the differences observed in the shape and texture of the bones of the skull in +A and −A dogs can also be found in the bones of the vertebral column. All delicacy of outline present in these complicated structures when normal disappears in the vitamin A-deficient animals and the bones become swollen and coarse. There seems to be little or no increase in the overall dimensions of each vertebra, but all the processes, including the arches and articular processes, are thickened to a greater or less extent. For instance, the wings of the atlas vertebra in one animal which received vitamin A were 2.2 mm. thick, whereas the thickness of the same region in its litter-mate not receiving vitamin A was 4.95 mm. Instead of the finely moulded edges seen in the normal atlas and axis vertebrae (Plate VIII), bulbous protuberances are usually found (Plate VIII c & d). It is unnecessary to describe these anatomical changes in individual vertebrae here, but their effect on the spinal cord and the spinal roots calls for some consideration. When the spinal cord is exposed by cutting through the vertebrae, it can be seen to be more closely

section of the bones in these two dogs, the internal space traversed by the spinal cord, its membranes and nerves is greatly reduced in the case of the −A dog. An idea of the size of the changes in the various areas of such cross-sections can be obtained from the above figures (Table 3) which represent a series of measurements made by weighing paper which just covered each part of the section and estimating each area by the weight of paper. The animals were *injected intra-arterially with Wittmaack's solution (1926)*, which tends to prevent shrinkage, so that the figures obtained probably approximate to those in life. These figures show (1) that the total area covered by the cross-sections of the comparable bones is not significantly altered in the −A animal, i.e. the vertebrae are not generally larger but are only locally thickened; (2) that the area of the lateral portions of the vertebra is increased in the −A dog; (3) that the main change in the −A dog is the reduction in space between the dura and the bone and to a less extent between the spinal cord and the dura. In brief, the spinal canal is much smaller in the −A animal than in the +A animal.

There is also a great reduction in the size of the spaces between the body and the wings of the vertebrae for the passage of the spinal nerve. In fact, on the left side of the section from the −A dog (Plate IXb) compression of these nerves and especially of the dorsal root ganglion can be seen between the wings and the body of the vertebra: this stage of compression, in comparison with the free space occupied by the corresponding nerves and ganglion in the normal animal, can be better appreciated in the higher power photomicrographs (Plate X). There is also some lengthening of the spinal nerves during their passage through the vertebrae of −A dogs, since the nerve roots pass more obliquely from the canal to the periphery than in the +A animals.

packed into the spinal canal in animals which were fed on −A diets than in those with a sufficiency of the vitamin.

Some of the changes in the bones and tissues immediately surrounding the spinal cord are represented in Plate IX, which are photomicrographs of sections through the 5th cervical vertebra of litter mates, one a +A animal and the other a −A animal. One of the difficulties in this work is that the alterations in shape of the bones, especially in complicated structures, make histological examination of identical positions difficult or impossible. For instance,

TABLE 3

Areas of various regions of vertebral cross-sections (5th cervical) of +A and −A dogs

Regions	Sq cm. actual area		Percentage of area	
	+A	−A	+A	−A
Spinal cord	0.338	0.336	8.05	8.01
Space between cord and dura	0.212	0.160	5.05	3.81
Space between dura and bone	0.668	0.384	15.9	9.15
Intervertebral disc and body of vertebra	1.428	1.547	34.0	36.88
Lateral portions of vertebra	1.371	1.614	32.65	38.48
Whole section of vertebra	4.200	4.195	100.0	100.0

Plate IX a & b are both roughly through the centre of the dorsal root ganglion, but owing to the bone dysplasia, the relative positions of other structures are altered and the photomicrographs are therefore not necessarily truly comparable. This difficulty is partially overcome in practice by examining the serial sections which have been made in most of these experiments, but it is not possible to publish the large number of photomicrographs necessary to show this. It will be seen, however, that, although there is not much difference in the total area covered by the cross-

The pressure exerted by the bones on the spinal nerves and the dorsal root ganglion in the animals fed on vitamin A deficient diets is undoubtedly largely or wholly responsible for the degenerative changes previously described in these nerves (see p. 39), although at the time of the experiments this cause was not appreciated. Attention, however, was drawn at that time to what seemed a curious fact namely, that the ventral roots near the spinal cord generally escaped degenerative changes, whereas the nerve fibres of the dorsal roots in the same position were often degenerate. The reason for this difference in distribution of nerve degeneration now seems clearer. The nerve cells of the dorsal root ganglion are often damaged by the effects of bone pressure and their axis cylinders, both peripheral and central to the ganglion, therefore degenerate. On the other hand, even if the ventral roots are destroyed by pressure at the same spot, the fibres central to the lesions will escape degeneration since their ganglionic origin is in the ventral horns of the spinal cord. The fibres of the ventral spinal nerves peripheral to the compression may, however, be destroyed. There is certainly much degeneration in the sciatic nerve and other peripheral nerves of spinal origin, but whether the efferent as well as the afferent fibres are affected is not known.

Another observation made in the earlier work was that all the spinal nerves in a given −A dog did not suffer equally (p. 66). Even at the same level of the vertebral

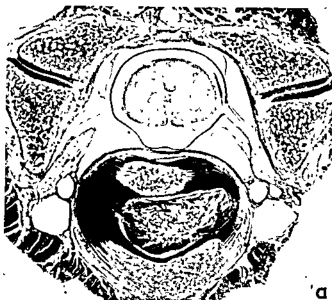
PLATE IX

Photomicrographs (× 5) of transverse sections through the 5th cervical vertebrae and the spinal cords of two litter-mate dogs of the same age.

(a) +A diet

(b) −A diet.

Note· Reduction in size of the vertebral canal and compression of the dorsal root ganglion in the −A dog (b) as compared with (a), also the alteration in shape of the dorsal surface of the intervertebral disc in (b).



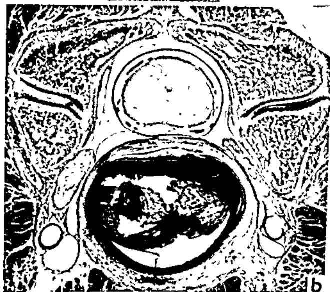


PLATE IX

in illustration) are not subjected to the same pressure effects as those on the left, so that the latter are more destroyed than the nerves of the right side.

3. Brain and Spinal Cord in Rabbits, Ferrets, and Rats

The bone changes so far reported have referred mainly to dogs but other young animals suffer similar changes in A-deficiency. The situation of greatest bone change varies from species to species, probably due to different rates of growth of the individual bones and various relative times of starting the A-deficient diet. A short survey of the changes seen in the species examined in this laboratory follows.

Rabbits. Macroscopic and microscopic examination of the skulls of A-deficient rabbits reveals large overgrowths around the IInd, Vth and VIIIth nerve foramina. The picture, indeed, is very similar to that which has been described for the dog. These overgrowths in rabbits reported in 1938 were confirmed by Perlman and Willard (1941) in regard to the labyrinthine capsule and its compression effect on the VIIIth nerve.

Rats. Loch (1939) and Wolbach and Bessey (1941) confirmed the bone overgrowth around the VIIIth nerve and on the surface of the labyrinthine capsule which had previously been described. Other bone abnormalities are found in these animals, notably in the vertebrae and some of the foramina of the Vth nerve system, and labyrinthitis has also been seen. The general question of bone lesions in —A rats is discussed fully in relation to Wolbach's work on pages 133–140.

Ferrets have not been examined for nerve degeneration, but their behaviour (p. 17), when suffering from A-deficiency, together with the obvious compression of the nervous system by abnormal bone, leaves no doubt that such

canal, sometimes the spinal nerves on one side escaped destructive changes while those on the other side suffered.

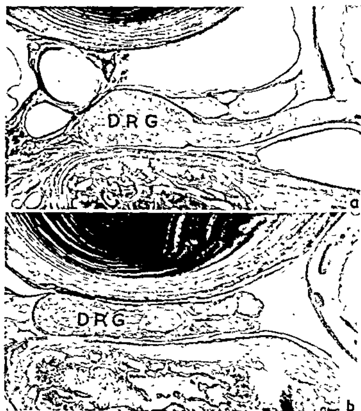


PLATE X

Photomicrographs (× 12) of the dorsal root ganglia of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Note: Compression of ganglion (D R G.) between body and lateral processes of vertebra in (b) as compared with (a).

The reason for this difference can now be understood. In Plate IXb, for instance, it will be seen that the spinal nerves and dorsal root ganglion of the right side (left side

b & c), which causes a severe distortion of the cerebellum and to a less extent of the cerebrum.

4. Summary

It has been shown in this chapter that in young dogs brought up on diets deficient in vitamin A:

1. The brain and spinal cord are more closely packed into the cranial cavity and spinal canal respectively than in litter mate +A dogs.

2. Malformation of certain skull bones causes compression and distortion of the brain and especially of the cerebellum and brain stem.

3. It is undoubted that changes of intra-cranial pressure are produced by vitamin A deficiency; the pressure of the cerebro-spinal fluid in the cisterna magna is increased, and a condition of slight but definite internal hydrocephalus is often found.

4. Increased pressure from the 3rd ventricle, together with overgrowth of the posterior clinoid process, causes compression of the pituitary body.

5. No evidence of interference with the blood supply to the brain has been obtained, but it is possible that in positions of great bone malformation, for instance, near the posterior fossa and cervical region of the cord, some restriction may occur.

6. The overall size of the vertebrae is similar to that of the +A dogs but, due to the thickening of the walls, the space occupied by the spinal cord and roots is reduced. Direct bone pressure is exerted on the dorsal root ganglia, which can be seen to be squeezed between the body and the wings of the vertebrae.

Abnormalities of the bones surrounding the brain and spinal cord similar to those seen in the dog have been described in vitamin A-deficient rabbits, ferrets and rats.

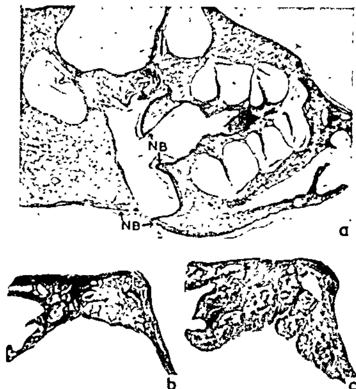


PLATE XI

(a) Photomicrograph (× 9) of the labyrinthine capsule of an adult dog (aged 30 months when first given −A diet), after having received the deficient diet for 15 months

Note: New bone (NB) near the modiolus and over the surface of the capsule.

(b) and (c) Photomicrographs (× 4) of the supraoccipital bones of two litter-mate ferrets of the same age.

(b) +A diet.

(c) −A diet.

Note: The bone in (c) is much larger than that in (b), showing that in the ferret overgrowth and actual laying down of extra bone as distinct from lack of absorption occurs in some situations.

degeneration must be present. Perhaps the most obvious of the bone changes seen in the skull of the −A ferret is the large overgrowth of the supra-occipital bone (Plate XI

nerve fibres traverse a single passage in the cribriform plate, but in the −A they seem to be broken up into smaller

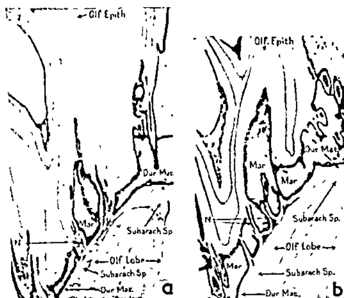


Fig. 23. Drawings showing the passage of the olfactory nerve through the cribriform plate of two litter-mate dogs of the same age

(a) +A diet

(b) −A diet

Black areas represent calcified bone

Note. In the −A dog (b)

(1) Swelling of bones with enlargement of marrow cavities (Mar.),

(2) Constriction of foramina of cribriform plate causing compression of olfactory nerve bundles (N);

(3) Enlargement of subarachnoid space;

(4) Fewer nerve fibres crossing the subarachnoid space;

(5) Outer layers of olfactory lobe thinner.

bundles by the encroaching and irregular bone over-growth; some of the bony passages are so narrow that even these smaller nerve bundles are pinched.

If high power photomicrographs of sections of the

Chapter VI

BONE OVERGROWTH AND CRANIAL NERVE DEGENERATION

Having seen in the previous chapter the effect of the malformed skull bones in −A animals on brain shape, it was decided to prepare serial sections of cranial nerves and the surrounding bones in normal and vitamin A-deficient dogs and to trace the course of each nerve in relation to any change in bone shape which might be found in the −A animal. Some of these results will now be described; they show that bone dysplasia can be regarded as the main cause of the extensive degeneration found in some cranial nerves.

1st Nerve (olfactory)

In the −A dog the cribriform plate is swollen, with enlargement of the marrow spaces between the limiting plates which, however, may not themselves be thickened (Fig. 23, compare a and b). Not only are the marrow spaces increased in that part of the plate which is adjacent to the brain tissue, but they are also enlarged in the projections of the plates into the folds of the olfactory mucous membrane, so that, whereas in the +A animal the bony trabeculae entering these projections may only appear as spicules of bone with small marrow spaces, in the −A animal a shell of bone with a large marrow space is often seen. These facts are demonstrated in Fig. 23, which also shows that the enlargement of the cribriform plate does not greatly increase the average distance to be traversed by the olfactory nerve fibres, but reduces the size of the nerve bundles which pierce the plate at any one point. In the +A animal a number of bundles of non-medullated

mucous membrane do not show any great change in $-A$ animals, although the cell layers may be irregular in appearance and the nuclei are sometimes nearer the surface than in $+A$ animals. The cells remain columnar in shape and do not become squamous or keratinized. The bipolar olfactory nerve cells in the mucous membrane, with their hairlets, may be reduced in number, but as yet no definite change in individual cells has been observed. As the relative number of receptor cells is known to vary in different parts of the normal olfactory mucous membrane, even the question of reduced numbers is not certain.

The behaviour of the animals and the histological appearance of the olfactory apparatus above described suggest that the effect of feeding on $-A$ diets is to reduce the number of olfactory nerve fibres and thereby the sense of smell. As previously stated, these dogs often run about with their noses near the ground sniffing vigorously, as if they were attempting to make up for their deficient smelling power. As the sense of smell is normally well developed and of great physiological importance in dogs, this excessive sniffing is the behaviour that might be expected.

IInd Nerve (Optic)

Reference has previously been made to the degenerative changes in the optic nerve and retina in $-A$ puppies (see p. 56), and evidence will now be given of the manner in which the nerve may suffer compression and stretching by bone overgrowth.

Whereas in a normal dog the nerve, as it passes through the optic foramen, lies in loose connective tissue, in a $-A$ animal, due to the encroaching bone, it is closely surrounded and in some advanced cases is gripped tightly and compressed (Mellanby, 1943). The dura mater is usually thickened in A deficiency and, possibly because it does not easily adapt itself to the shape of the locally

olfactory nerves of a $-A$ animal be made before, during and after passing through the cribriform plate, it will be seen from the bunching together of the nuclei of the sheaths that the fibres are much compressed during their actual passage through the bone towards the brain. In spite of the pressure, however, these nerves are generally not completely destroyed, although it may be that other bundles of nerves or some nerves in any bundle have degenerated and disappeared.

In this, as in other situations, changes are sometimes better observed in the dura mater and subarachnoid space in $-A$ animals. The dura mater, which accompanies the nerve bundles in part of their passage through the cribriform plate, is thickened and may add to the pressure effect on the nerve bundles referred to above. The subarachnoid space of the $+A$ animal contains much nerve tissue and some other connective tissue elements, whereas in the $-A$ animal this space seems relatively free from nerve and connective tissue. There is, however, one other factor to be considered in this connection, namely the raised intracranial pressure in some of these $-A$ animals (see p. 80). Increased pressure in the subarachnoid space may affect both the olfactory fibres crossing it and the surface layers of the olfactory lobe itself. That there is destruction of nerve fibres of the olfactory lobe is evident, since in some of the experiments myelin degeneration has been observed in this position. Moreover, in $-A$ animals the superficial layer of the olfactory lobe of the brain is often thinner, so that the glomerular layer of cells is nearer the brain surface than in control animals (Fig. 23b). This again may be partly due to compression associated with increased intracranial pressure, and partly to the disappearance of many olfactory nerve fibres normally entering the brain in this position.

The epithelial or sustenticular cells of the olfactory

at this point is indicated by the facts that: (1) the cross-section of the nerve is oval instead of round, and (2) the blood vessels in the connective tissue are smaller than in the +A animal. Owing to the bone dysplasia described, the optic foramen in the −A animal is longer and more tortuous than that in the +A.

The appearance of the nerve as it emerges from the foramen and passes over the surface of the base of the skull towards the optic chiasma can be seen in other drawings (Fig. 25). The bone enlargement in the −A animal (series b) again stands out prominently and is seen to be due to the increase in *cancellous tissue*. The compact bone is not as abundant as in the normal animal (series a), so that the total calcified bone of the −A animal is not nearly as great as its increased mass would suggest. It will be noticed that, whereas the optic nerve in the normal animal runs along a gentle depression in the sphenoid bone from the optic foramen (lowest figure) towards the optic chiasma (top figure) and is round in cross-section, the depression in the −A animal is much deeper and the nerve is compressed, as can be seen by the distorted shape of its cross-section (series b). These are the main bone changes which appear directly to affect the optic nerve.

Besides the direct mechanical squeezing of the optic nerve by bone and dura mater in the −A dogs, it is probable that the raised intracranial pressure (see p. 80), also helps in its destruction. Clinical experience in man teaches that the optic nerve is particularly susceptible to mechanical pressure in any part of its course, and when a nerve fibre is thus injured degenerative changes do not follow the Wallerian law, but the whole neurone may be destroyed. It would be expected, therefore, that in these −A dogs the bone overgrowth, which has been shown to exert undue pressure on the nerve during its bony passage, as well as the raised intracranial pressure acting

hypertrophied bone, it may be in a state of increased tension at points where nerves pass through it, and thereby contribute to the nerve compression.

Bone overgrowth around the optic nerve in a −A dog can be clearly seen by comparing serial sections from +A and −A dogs. Fig. 24 represents drawings of sections through the nerve as it leaves the orbit on its way to the



FIG. 24. Drawings of coronal sections of the optic nerve near the orbit in two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Black areas represent calcified bone. Diagonally shaded areas = various portions of IIIrd, IVth, Vth and VIth nerves. E.M. and similarly shaded areas = eye muscles.
Note. In (a) both openings of the bony canal through which the optic nerve passes from the orbit to the chiasma can be seen; in (b) the nerve is almost surrounded by bone and, owing to the lengthening and twisting of the canal by the bone overgrowth, only one opening is evident

brain. In the −A dog (Fig. 24b) the fatty marrow spaces are enlarged, thus increasing the bulk of bone, which almost completely surrounds the nerve in this position, leaving only a small opening into the orbital cavity and none into the cranial cavity. In the corresponding section of the +A animal (Fig. 24a) the opening into the latter is seen. Here the connective tissue surrounding the nerve is loose, whereas in the −A litter-mate it is tightly packed. That there is some compression of the nerve in the −A animal

on the nerve within the cranial cavity, would cause degenerative changes in the nerve.

Moore, Huffman and Duncan (1935 a, b) described in calves blindness of a nutritional type resulting from atrophy of the optic nerve where it passes through the optic foramen. This atrophy, they suggested, was caused by improper development of the foramen. They did not notice any bone exostosis but said that 'the bony canal gave more the appearance of having had pressure applied from above, which caused it to become smaller as growth proceeded'. After the publication of the paper on the production of excessive labyrinthine bone in vitamin A deficiency and the destruction of the VIIIth nerve (Mellanby, 1938), Moore (1939) came to the conclusion that carotene deficiency was the cause of nyctalopia, papillary oedema and permanent blindness in calves and, although he again ascribed the nerve degeneration to bone pressure due to stenosis of the bony canal, he thought it was 'difficult to associate vitamin A deficiency with any bony malformation', and that increased intracranial pressure probably accounted for the abnormal bone development. Similar eye conditions are found in −A dogs (Mellanby, 1943). The earliest retinal change observed in dogs by the ophthalmoscope is an alteration in the colour of the tapetum lucidum, which often loses its blue component. The blue band contiguous with the tapetum nigrum becomes green and the green coloration gradually changes to yellow from its upper boundary downwards to the tapetum

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Fig. 25 Drawings of sections of three corresponding regions of the optic nerve between the orbit (lowest fig) and the optic chiasma (top fig) in two litter-mate dogs of the same age
(a) +A diet.
(b) −A diet.
Black areas represent calcified bone.
Note: In (b) series greatly enlarged cancellous bone as compared with (a) series and alteration in shape of the nerve due to compression.

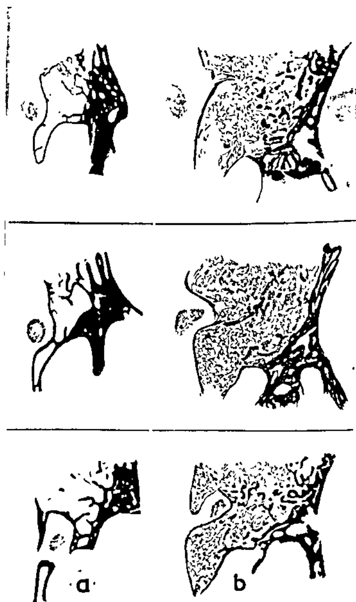


FIG. 25

IIIrd, IVth and VIth Nerves (Oculomotor, Trochlear and Abducens)

These nerves, in which no degeneration has been found (p. 39), together with the first branch of the Vth, pass through the superior orbital fissure, where there is relatively little bone change in A deficiency, so that the nerves are not compressed to any great extent. The only nerve passing through this fissure which is regularly found to be degenerated is the first branch of the Vth, which will be considered in the next section with the trigeminal system as a whole.

Allusion has been made above to the distorting effect of the dura mater on the optic nerve. A similar effect can sometimes be seen on the IIIrd nerve of −A dogs. This nerve is normally slightly bent at the point where it passes through the dura mater into the cavernous sinus, but in −A dogs the distortion is exaggerated. It may be noted that in the one case in which the region of this distortion was examined histologically, a few degenerated fibres were found. Since, however, there is seldom much degeneration in the IIIrd nerve in −A animals, and indeed this applies to all the motor nerves of the eye, the mechanical distortion produced in this region by the dura mater alone does not apparently interfere to any great extent with the structure of the nerve fibres or with their function.

Vth Nerve (Trigeminal)

Degeneration of the Vth nerve and its branches in −A animals was described in Chapters II and III, where it was shown that there was often a relationship between degeneration of the 1st division of the Vth nerve and xerophthalmia.

As previously described (p. 78), macroscopic examination of the skulls of −A dogs reveals large overgrowths of the bone surrounding the Vth nerve system. The petrous

nigrum. Ultimately the whole of the tapetum lucidum is yellow and the tapetum nigrum sometimes seems rather a darker brown than in the normal.

The early changes in the tapetum are soon followed by pallor of the disc, which may be associated with protrusion of about half a diopter. Swelling of the optic disc to a degree which can be described as definite papilloedema takes place slowly after these initial changes, and may in some of the dogs reach as much as 8 diopters. In the early stages the pale disc, when viewed with the ophthalmoscope, has a sharp outline. Later the edge is blurred and irregular, in some cases with apparent extensions of the disc along the vessels, especially on the nasal side. Photomicrographs of sections of the optic nerve and retina of +A and −A dogs are shown in Plate III, (p. 38). The papilloedema (Plate III f) is not severe in this −A animal, but is of a degree which is easily and regularly produced. As will be seen in Chapter IX many of the retinal changes including papilloedema are reversible if vitamin A is added to the deficient diet at an early stage.

An attempt was made in the course of this work to see whether there was any simple relationship between the degree of abnormality of the optic disc and the degree to which overgrown bone compressed the nerve as it entered the cranial cavity from the optic canal, but the evidence was inconclusive. This may possibly be regarded as supporting the view that a factor other than pressure of bone and raised intracranial pressure is involved. This other factor may be the direct action of vitamin A deficiency on the retina, a change which begins to manifest itself early in the experimental period by bleaching of the tapetum lucidum (p. 103). At a later stage bone pressure on the optic nerve and the increased intracranial pressure become effective and produce a condition of papilloedema.

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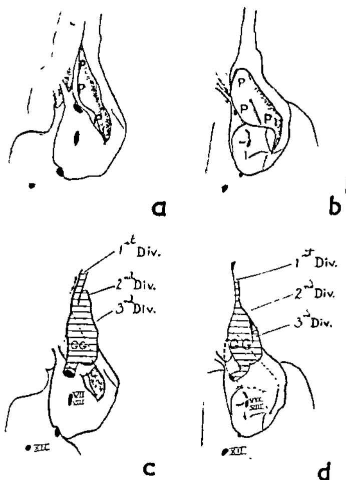


Fig. 26. Drawings of the petrous ridge (P) of the temporal bone in two litter-mate dogs of the same age. (a) and (b) before removal of the petrous ridge, (c) and (d) after removal to expose the Vth nerve and Gasserian ganglion.

(a) and (c) +A diet.

(b) and (d) −A diet.

Note: (1) Thickened bulbous ridge (PPP) in (b) as compared with (a).

ridge is swollen and blunted, the increase in size being in depth and width rather than in length, as can be seen in Fig. 26. When the bone of the petrous ridge is removed, it will be seen that overgrowth has greatly affected the course of the Vth nerve. In the −A animal (Fig. 26d) the central branch of the nerve is bent and passes from the bony foramen towards the pons in a more mesial position. In the normal animal (Fig. 26c) this branch of the nerve runs straight from the Gasserian ganglion to the pons.

Fig. 27 shows drawings of sections, as far as possible comparable, through the trigeminal nerve and petrous bone of a +A and −A dog. Owing to the great overgrowth of the petrous ridge, it is impossible to obtain really comparable sections as the relative positions of the Gasserian ganglion and Vth nerve foramen may vary. It has been usual, therefore, to compare sections in relation to the foramen lacerum. In the +A animal the nerve is cut transversely to its length and occupies a relatively wide space in the bone (Fig. 27a, i, ii), whereas the twisting of the nerve in the −A animal is such that it is seen to be cut parallel to the plane of the section (Fig. 27b, i, ii). The overgrowth of the petrous ridge has also encroached on the space occupied by the nerve and has compressed it. A small portion of bone (X) can be seen immediately beneath the nerve in Fig. 27a (i, ii), but in the −A animal (Fig 27b, i, ii and iii), this piece of bone (X) is much greater in size and, together with the overgrowth of the petrous ridge, has caused considerable compression of the Vth nerve. Fig 27a (vi) shows the normal appearance of the Gasserian ganglion in relation to the bone surrounding it. In Fig. 27b (vi), a drawing of the section through the same region in a −A animal, the Gasserian ganglion is com-

(2) The twisting, due to bone overgrowth, of the central branch of the Vth nerve in (d); in the normal (c) this branch is straight.
(3) The reduced size and folded wall of the VIIIth nerve foramen in (d).

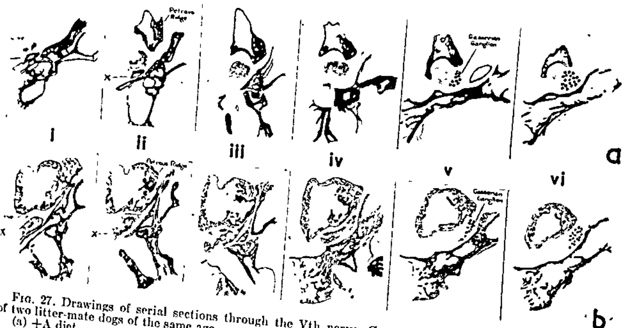


Fig. 27. Drawings of serial sections through the Vth nerve, Gasserian ganglion and surrounding bone of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Black areas represent calcified bone.
Note: (1) Overgrown petrous ridge in (b) as compared with (a).
(2) In (b[i, ii and iii]) the nerve is cut longitudinally, whereas in (a[i, ii and iii]) it is cut transversely; this is due to twisting of the nerve in (b).
(3) In (b[v]) the ganglion can be seen to be compressed between the petrous ridge and the apex of the petrous bone, as compared with the ganglion in (a[v]).

pressed and elongated by the great overgrowth of the petrous portion of the temporal bone. It will also be seen that the depression of the internal surface of the petrous ridge into which the ganglion fits has become flat in the *−A animal* (Fig. 27b, vi). Although the Gasserian ganglion is squeezed between the apex of the petrosal portion of the temporal bone and its petrous ridge, the nerve cells do not show the elongation that might be expected; they do, however, undergo chromolytic and other degenerative changes which may be due to the mechanical pressure of the abnormal bone.

In cases where the effects of the A-deficient diet are less, the overgrowth of the petrous portion of the temporal bone may not be so obvious and twisting of the central branch of the Vth nerve may not occur. Fig. 28 b is a section through the foramen lacerum of such a dog and compares with a section from a control dog of the same age (Fig. 28a). The overgrowth of bone in the −A animal is again obvious, the additional bulk being made up of loose cancellous bone at the expense of the compact bone seen in the control animal (Fig. 28a). It will be seen also that the nerve is deeply placed due to the bone hypertrophy. *The deformity of this nerve in Fig. 28b is obvious;* it is now triangular in cross-section in keeping with the altered shape of the bony canal.

When the Vth nerve has emerged from under the petrous ridge in its passage towards the periphery, it assumes a more normal appearance, since the bone overgrowth produced by the vitamin A-deficient diets is less prominent, although still definite. For instance, if *sections passing* through the foramen ovale are examined, the sphenoid bone is seen to be thickened and the foramen is narrowed, but not sufficiently to press on the mandibular branch of the Vth nerve and distort its shape. The sphenoid bone *surrounding the foramen rotundum, through which the*

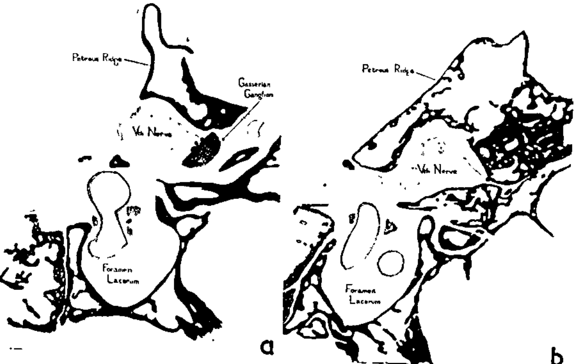


FIG. 28. Drawings of sections through the Vth nerve and surrounding bone at the level of the foramen lacerum in two litter mate dogs of the same age.

(a) +A diet.

(b) −A diet.

Black areas represent calcified bone.

Note: In (a) the nerve and part of the ganglion are surrounded by loose tissue, whereas in (b) no ganglion cells are seen and the shape of the nerve is altered by overgrown and encroaching bone which almost surrounds it.

maxillary branch passes, is also slightly thickened and the foramen narrowed, but again there is no evidence of bone pressure on the nerve.

The ophthalmic branch of the Vth nerve, together with the IIIrd, IVth and VIth nerves, passes through the superior orbital fissure towards the orbit. The bone is thickened in this position, but not so as to reduce the fissure, and there is no evidence of bone pressure on the nerves traversing it. It is possible that these nerves are affected by increased intracranial pressure, suggestive evidence of which is obtained by examining the blood vessels running with them. In the cases examined they are smaller than the corresponding vessels in the same relative position in normal animals. The connective tissue surrounding the *nerves and vessels seems also to be compressed and not* only to occupy a smaller space but to be denser in the vitamin A-deficient dogs than in the control animals. This possible effect of increased pressure on blood vessels in the brain may be of importance and requires more detailed study.

One other mechanical effect of bone overgrowth on the Vth nerve, which may be of some importance, is its increased length in vitamin A-deficient animals; this becomes more apparent when serial sections of the nerve and its surrounding bone in comparable +A and −A dogs are examined. It may involve actual stretching, or the nerve may simply lengthen without any increase in tension. It was thought at first that the lengthening of the nerve might *itself cause degeneration, but a similar lengthening* produced by thickening of bone was found to occur in other cranial nerves, including the IXth, Xth, XIth and XIIth, with little or no degeneration, and it is probable that the degeneration suffered by the Vth nerve is not due to this cause.

It seems from the foregoing account that the great

susceptibility to destructive change of the more purely sensory divisions of the Vth nerve in −A dogs can be accounted for by overgrown bone pressing on the Gasserian ganglion.

VIIth Nerve (Facial)

It has been shown that the motor cranial nerves are relatively little damaged in −A animals in which the sensory cranial nerves are severely affected, due no doubt in some measure to the different positions of their cells of origin. Of the motor cranial nerves, probably the VIIth is most often affected whilst, of the sensory cranial nerves, the adjacent VIIIth is most often destroyed.

In its passage from the central nervous system to its exit from the skull, the VIIth nerve may be roughly divided into five parts: (1) the portion from the point of issue from the brain stem to the internal auditory meatus; (2) the portion running close to the VIIIth nerve within the internal auditory meatus; (3) a short portion which leaves the internal auditory meatus near the modiolus and connects with the geniculate ganglion. The nerve here is within the facial canal which passes in an antero-lateral direction; (4) a part which, beginning at the geniculate ganglion, bends through an angle of practically 120° and then passes posteriorly through the facial canal in the upper wall of the tympanic cavity; and (5) the portion which passes through the stylomastoid foramen.

Taking these parts separately, it is obvious that the first part passing from the brain stem to the internal auditory meatus, will not be affected directly by bone overgrowth and, in fact, will only be subjected to the same increase of intracranial pressure as may influence any part of the central nervous system in −A animals. The second part of the nerve will be subjected to the same conditions as the VIIIth nerve in this position. Bone overgrowth in

this neighbourhood of the VIIIth nerve in $-A$ animals has been described above (p. 78). The internal auditory meatus is often greatly lengthened and twisted by overgrown periosteal bone of the layrinthine capsule but, though constricted, it has always been patent, as seen in serial sections. If there is ever complete destruction of the VIIth nerve due to bone overgrowth at this part of its passage, it must be very rare. Indeed, it is the new bone adjacent to the helix and the spiral ganglion rather than that around the internal auditory meatus which is responsible for the severe changes seen in the VIIIth nerve. The *VIIth nerve does not reach the modiolus, but turns away* from the VIIIth nerve to enter the facial canal some distance before the helix is reached. Thus, although the VIIth nerve, when passing through the internal auditory meatus, is liable to suffer some stretching and compression by overgrowth of periosteal bone, there is no evidence that it suffers severe destructive changes in this position.

In the third part of its course through the facial canal, the VIIth nerve is again liable in $-A$ animals to be compressed by the partial closure of the canal, which is lined by layers of new formed bone. The resultant narrowing of the canal does not proceed to complete occlusion, but it may be sufficiently great to press on the geniculate ganglion and elongate the cells in a way similar to, but to a less degree than, that seen in the dorsal root ganglion and in Scarpa's ganglion in $-A$ dogs. In spite of the elongation by pressure, the cells of the geniculate ganglion from which the sensory fibres of the VIIth originate, usually suffer but little destructive change, and their Nissl's granules and nuclei generally seem normal or nearly so. In severe cases, however, there is definite degeneration of some of the cells but it is obvious from their appearance and that of the canal that they can withstand a good deal of pressure and distortion without degenerating. It may be that, when

degenerating fibres have been found in the VIIth nerve, they are fibres having their cells of origin in the geniculate ganglion.

In the fourth part of its course, where the VIIth nerve runs through the facial canal in the upper wall of the tympanic cavity, the compression due to bone overgrowth may be severe. In several instances the nerve has been seen to be compressed to a thin ribbon, but complete occlusion of the canal has never been observed even in the most severely affected cases; nor, indeed, have the blood vessels which pass with this part of the nerve appeared unduly narrowed.

The fifth part of the nerve now issuing from the skull wall through the stylo-mastoid foramen is unrestricted by bone overgrowth and the passage usually appears to be quite normal. It seems, therefore, that the VIIth nerve is liable to be affected by bone overgrowth in the second, third and fourth parts of its course, and it is in these positions that degenerating nerve fibres may sometimes be found. Even in severe cases of A deficiency, however, the number of such fibres is relatively small.

Reference may be made here to two nerves which pass into the VIIth namely, the greater superficial petrosal passing from the spheno-palatine ganglion on the second division of the Vth nerve to the geniculate ganglion on the VIIth nerve and the auricular nerve which passes from the jugular ganglion of the Xth to join the VIIth near the stylomastoid foramen. Both these nerves may be compressed in —A animals, especially the greater superficial petrosal which, in advanced cases, may be pressed to a ribbon shape by the encroaching bone. In spite of this distortion, however, there may be only a few fibres in the nerve showing degenerative changes. The compression of the auricular branch of the Xth nerve is not so great, and here again there are usually only a few degenerated fibres.

Facial paralysis has not been seen in these experimental animals, and, in spite of the severe constriction of the nerve sufficient degeneration to justify paralysis has not been found. In human beings also the VIIth nerve is known to be very resistant to pressure. The absence of facial paralysis in dogs and man in spite of compressed VIIth nerves, emphasizes the fact that some nerve fibres can withstand a surprising amount of mechanical pressure and distortion without loss of function (see p. 120).

VIIIth Nerve (Auditory)

The effect of vitamin A deficiency on this nerve has been described fully in Chapter IV.

IXth, Xth and XIth Nerves (Glosso-pharyngeal, Vagus and Accessory).

These nerves in which no degeneration was found (p. 39) pass together from the cranial cavity through the jugular canal, the walls of which are formed by portions of the temporal and occipital bones. Both these bones are thickened in −A dogs, but in spite or possibly because of the fact that two bones are involved, the jugular canal is only slightly narrowed, the main effect being the lengthening of the canal from the base of the brain to the wall of the tympanic cavity. At this latter point the jugular canal turns slightly and passes over and round the wall of the tympanic cavity. It might be expected that, in passing over the tympanic cavity and under the thickened basi-occipital bone, the nerves would be subjected to increased pressure. This, however, does not appear to be the case. It seems probable that constriction of the canal is avoided at this point because the space occupied by the thickened occipital bone is obtained at the expense of the tympanic cavity, which is smaller and surrounded by thicker walls in these than in the +A animals.

The only change in diameter of the canal appears to be near the internal end. Here the bone is sometimes seen to be folded and the ganglia on the nerves seem to fit more tightly in the canal. Changes in these ganglia when examined either by naked eye or histologically are very slight, even in severe vitamin A deficiency. Generally speaking, the only mechanical change observable in −A animals is the lengthening of the nerves owing to bone hypertrophy, and there is no conspicuous pressure of bone on either these nerves or their ganglia. Examination of the nerves by Marchi's method shows that very few degenerated fibres are present in those parts which are within the jugular canal. After the nerves have emerged from the canal a few more such fibres can be seen.

XIIth Nerve (Hypoglossal)

In −A animals the hypoglossal canal, which passes through the basi-occipital bone, is lengthened because of the thickening of the bone, but apart from this and an occasional fold of bone, which occurs in severe −A cases, the changes are slight. The hypoglossal nerve shows a similar lengthening, but no other mechanical interference arises, and when examined by a modified Marchi technique, no significant degeneration is seen in the nerve even in cases of severe A deficiency.

An outstanding fact, under the conditions of these experiments, is that the IIIrd, IVth, third branch of the Vth, VIth, VIIth, IXth, Xth, XIth and XIIth nerves tend to escape destructive changes.

Summary

It has been shown in this chapter that when young dogs are brought up on diets deficient in vitamin A,

(1) Local overgrowth of certain skull bones causes compression, twisting and lengthening of most cranial nerves,

some of which show large degenerative changes. These changes are intensified if, as happens in the VIIIth and Vth nerves, the ganglion cells are also subject to the pressure of overgrown bone.

(2) Destructive changes are largely confined to the sensory nerves, the motor cranial nerves for the most part escaping.

(3) Cranial nerves, especially those with motor function, such as the VIIth can often be compressed, lengthened and twisted as the result of bone dysplasia without degenerating.

(4) In the experiments described the nerves most affected in diminishing order are usually as follows: (a) Cochlear and vestibular divisions of the VIIIth (auditory) nerve, especially the former, (b) Vth nerve (trigeminal), first and second branches especially, (c) IInd nerve (optic), (d) Ist nerve (olfactory).

(5) Degeneration in the optic nerve probably results from direct pressure of overgrown bone and from increased intracranial pressure; possibly there may also be a primary degenerative change beginning in the retina itself. The optic atrophy associated with bleaching of the tapetum may be a direct effect of A deficiency on retinal cells, while papilloedema, due to bone dysplasia and increased intracranial pressure, is superimposed later.

(6) Whereas the internal ends of foramina in the skulls are generally stenosed, with folded outlines due to bone overgrowth and not to cessation of growth, the external openings are not usually smaller than normal.

Chapter VII

MALFORMATION OF BONE AND ITS EFFECTS

It will be seen from Chapters IV to VI that the investigation had now come full circle. It began with an observation, made while studying the aetiology of rickets, a disease of bone, to the effect that a deficiency of the fat-soluble vitamin A produced incoördination of movement as well as poor bone calcification. When this original vitamin A complex was later separated into vitamins A and D, it became obvious that a deficiency of the former was responsible for the incoördination and of the latter for poor bone calcification. A close study was next made of the distribution of degeneration in both the central and peripheral nervous systems in A deficiency and this led the investigation back to a bone abnormality, for it now became clear that the nerve defects responsible for incoördination of movement were themselves primarily due to abnormal growth of bones, which, instead of protecting the nervous system, as was their function, tended to damage it.

Although in Chapters IV to VI it was shown that the changes in the shape and texture of bones near the central nervous system in A deficiency accounted for much of the nerve degeneration, these observations not only suggested other problems of their own, but also made it necessary to consider, in the light of these newly discovered facts, some of the questions which were left in the air in Chapter II and which at the time could not be fitted into any reasonable scheme. It is opportune, therefore, at this stage, to re-consider some of the earlier difficulties and the first of these will be the different respective susceptibilities of the motor and sensory nerves to degeneration. It will be remembered that, although the sensory and afferent nerves

were much more affected in vitamin A deficiency than motor and efferent nerves, the differentiation was not quite clear cut, some afferent fibres, as for instance those of the vagus, escaping damage, whereas occasionally some fibres of the motor nerves were degenerated.

1. Variation in Susceptibility of Nerves to Pressure

It is probable that the chief reason for the difference between sensory and motor nerve susceptibility to damage is the presence in the course of the former of ganglia outside the central nervous system and the pressure to which the cells in these ganglia are subjected by overgrown bone. Since the cells of origin of motor nerves are within the central nervous system, they escape this form of direct pressure and only the nerve axons can be squeezed. For *instance, in the spinal nerves the dorsal roots, the ganglia* of which are exposed to the effects of bone pressure, usually show severe degeneration at a time when the ventral roots, whose axons pass through the same region, remain normal. A similar difference is seen in the Vth nerve system, for the Gasserian ganglion is compressed by the overgrown petrous bone, and degenerative changes are found in all three divisions. The mandibular division, however, contains a much smaller proportion of degenerated fibres than either the maxillary or the ophthalmic division. Here again it is probable that the motor fibres of the mandibular division escape destructive changes because their cells of origin are within the central nervous system and therefore are not subjected to severe compression, although their axons when within the petrous bone are exposed to increased pressure, as is the Gasserian ganglion. That this is, however, not the whole explanation of differences in the susceptibility of motor and sensory neurones to pressure is clear from examination of the vestibular and cochlear divisions of the VIIIth nerve.

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part of the nervous system escape damage. It is indeed all the more surprising since the underlying central nervous system in this region also shows most degeneration. Thus the IXth, Xth, XIth and XIIth nerves are seldom affected, either in their afferent or efferent neurones, and even the peripheral ganglia of their afferent fibres seem to escape. The nerves appear to be lengthened as they traverse the thickened bones, but evidently this lengthening does not harm them, perhaps because it is a relatively slow process. It seems possible from examination of the jugular foramen (see page 115), through which the IXth, Xth, and XIth nerves pass, that a foramen formed by two bones does not become as stenosed in A deficiency as a canal in one bone, and this may at least partly account for the lack of degeneration in these nerves when compared with the VIIIth nerve, which passes through a canal in one bone.

Attention has already been drawn (p. 101) to the fact that the optic nerve in A-deficient animals may show greater degeneration than would be expected from the degree of bone pressure exerted upon it. Early retinal changes have been observed in these animals and it is possible that a deficiency of vitamin A affects the retina independently of pressure on its nerve fibres. It has been seen (see note, p. 59) that the absence of vitamin A has an important direct effect on the visual purple cycle within the retina and it may be that still other cells and biological processes at present unknown are also directly affected.

In spite of the many questions that remain to be answered, it is probably true to say that the relative destruction of motor and sensory nerves in −A animals can be mainly related to three facts:

(i) The presence on the peripheral course of some afferent nerves of ganglia which are exposed to direct bone pressure, whereas the ganglia of motor nerves, being

Both are damaged by the bone overgrowth, the cells of Scarpa's ganglion often being malformed because of direct pressure (Plate VIc), and yet it is the cochlear division, the cells of which are *not directly compressed by bone*, which shows *the more degeneration*. This phenomenon was considered in detail in 1938 and authority was quoted to show that, in the mammalian internal ear, whereas pressure on any part of the neurone of the cochlear division caused *complete degeneration, similar pressure on the vestibular neurone only resulted in the type of degeneration expected* under the Wallerian law. It is also known from clinical experience that pressure of long duration on any part of the optic nerve causes degeneration both of the fibres of the nerve involved and of the associated ganglion cells in the retina.

Other nerves can apparently withstand a great deal of pressure, for the VIIth nerve of the vitamin A-deficient animals must be subjected to severe pressure effects when within the internal auditory meatus, but, as was reported in Chapter VI, degeneration is rare. This resistance of the *facial nerve has also been observed clinically in man*; for instance, Perlman and Willard (1941) have pointed out that the nerve "may be compressed to a microscopically thin ribbon on the capsule of the tumour and yet no facial paralysis is seen". It is possible that all nerves vary in their susceptibility to pressure, and if variations between the extreme examples given above do occur, they might well *explain the different degrees of degeneration* reported in the *cranial* and *spinal* nerves.

Considering the relatively big alterations produced by A deficiency in the shape of bones surrounding the posterior fossa, as compared with other bones in close proximity to the central nervous system, and the resulting abnormal shapes of the cerebellum, pons and medulla, it is surprising that a number of cranial nerves entering and leaving this

and mid-brain was thought to be due to the large numbers of degenerated fibres leaving the brain stem and entering the cerebellum. However, closer examination showed that the increase in nerve degeneration in the cervical region was so great that it was difficult to escape the conclusion that some harmful influence was specially affecting the dorsal roots of that region. This, of course, we know now to be true, for the bone abnormalities in the cervical vertebrae are greater than in other regions.

Other factors which may play a part in the greater degenerative nerve changes found in the brain stem from the lower margin of the pons to the lower cervical region are related to the cerebrospinal fluid and the blood supply. Here, however, we are dealing with a purely hypothetical question, because we have no knowledge of the rôles these fluids normally play in the maintenance of structure and function of the central nervous system. As shown in Chapter V, however, the amount of cerebrospinal fluid in A-deficient animals is diminished around some parts of the central nervous system; the closely packed lower brain stem and upper cord may greatly limit any free flow of fluid in the subarachnoid space, and this in turn may interfere with the nutritional supplies to the associated nervous tissue. In particular, it has already been pointed out (p. 80) how difficult it often is to obtain a sample of cerebrospinal fluid from the cisterna magna of A-deficient dogs. Whatever may be the direct cause of the increased nerve degeneration in this part of the central nervous system, it is undoubted that the region suffering greatest degenerative changes in A deficiency is that part surrounded by bone which is most malformed. As a counterpart to these results it is to be noted that where there is least overgrowth of the skull bones, i.e. in front of the optic chiasma, there is very little nerve degeneration in the brain. Generally

placed inside the nervous system, escape such pressure.

(ii) Variations in the degree of bone pressure to which different nerves are subjected and possibly to variations in intracranial pressure.

(iii) Variations in susceptibility of nerves to pressure effects, the evidence favouring the view that efferent neurones withstand pressure better than afferent neurones.

2. Distribution of Nerve Lesions within the Central Nervous System

It may be asked whether the bone overgrowth and malformation in the skull and vertebrae of —A dogs explain the degree and distribution of degeneration in the brain stem and spinal cord. The vertebral bone abnormalities will account for degenerated fibres having their origin in the dorsal root ganglia; but is there a satisfactory explanation of the excessive amount of degeneration in the cervical cord and the medulla compared with other parts of the central nervous system? Can also the degeneration of the second ascending neurones, such as those of the dorsal and ventral spino-cerebellar tracts, having their origin in Clarke's column cells, be explained by pressure from abnormal bone?

Even if there was no greater bone abnormality in the cervical than in other vertebrae, the larger number of degenerated ascending fibres in this region might only be a summation of the amount suffered by individual spinal roots. This ascending degeneration would continue into the medulla where it would be joined by severely degenerated VIIIth nerve fibres. This was the view put forward in 1935 to explain the specially great degeneration in these regions. The reduced amount of degeneration in the pons

diets, became very incoördinate and suffered severe nerve degeneration while adults, after many months or even years of the diets, developed relatively little ataxia and only slight nerve changes. Various explanations were considered, but now it is obvious that fully and correctly formed bones of adults will not be converted to a swollen and overgrown state as quickly as those in the process of growth and development. Bone overgrowth has, however, been seen on the surface of the labyrinthine capsule and within the internal auditory meatus of adult dogs fed for 15 or more months on the A-deficient diets, but in such cases the reaction is generally relatively slight (see Plate XI a).

Abnormal bone growth and subsequent pressure effects on the nervous system appeared, therefore, to fit most of the facts, both in young growing animals and in adults, but posed another question, namely as to how and why the abnormal bone growth occurred. The answer to this question is considered in Chapter VIII.

4. Bones Unrelated to the Nervous System

Although most attention has been given in these investigations to the question of abnormal growth of bone situated in close relation to nervous tissue, this does not mean that other bones are not affected by vitamin A deficiency. It will be seen in the next section that the long bones may be altered and, in fact, the changes in bone shape, and especially the abnormal moulding, probably affect to a greater or less degree all the bones of the body. For instance, some of the bones of the face, in particular the mandible, the malar bone and the zygomatic process of the temporal bone, are thickened and coarsened. Fig. 29 shows the malar bones of a normal and a vitamin A deficient dog. It will be seen that in the latter the bone extends upwards and encroaches to a larger degree on the lower margin of the orbit. This swelling below the orbit may give a −A dog a

speaking, therefore, the malformed bone hypothesis explains reasonably well the distribution and type of degeneration of the central nervous system.

Whether direct pressure on the central nervous system is ever great enough to account for the remarkably large amount of degeneration in the dorsal and ventral spinocerebellar tracts in −A animals, cannot be stated with certainty. It may be related to the severe destructive changes often seen in the cerebellum, especially in the Purkinje cells (p. 33), or it may be of transneuronal origin. In the first case the original seat of the injury would be at the cerebellum end of the spinocerebellar tract; in the second case, it would be at the spinal end, i.e. due to destruction of the primary neurone ending among Clarke's column cells. Whether nerve fibre degeneration can result from the first type of injury is not known to the author, but degeneration of transneuronal origin is certainly possible. Thus Clark and Penman (1933) used the fact that cells atrophy in the lateral geniculate body of monkeys after retinal lesions to map out the localization of the retina in this part of the nervous system. Also the cells of the dorsal horn of the spinal cord may degenerate on removal of dorsal spinal cells supplying the primary neurone fibres. The same applies to the motor side. It is said that anterior horn cells may degenerate in cases of cerebral tumours affecting the pre-central convolution. All these are instances of degeneration of a secondary neurone following destruction of the primary connecting neurone and it may well be that the spino-cerebellar tracts degenerate owing to destruction of primary neurone fibres starting in the dorsal spinal ganglia.

3. Relative Susceptibility of Young and Adult Animals

Another problem in the early days of this work was to explain why young animals, when fed vitamin A-deficient

lesions in −A animals, a fuller investigation into bone growth and shape was begun. Anatomical examination of the skull, vertebral column and the central nervous system revealed that the general effect of vitamin A deficiency, when reduced to its simplest terms, was a crowding of the latter into a bony box which was too small to accommodate it. The overall size of the skull and vertebral column showed only small differences quite inconsistent with the crowding of the brain. A closer examination, however, showed that there were significant alterations in the thickness of bony walls as well as in details of finer moulding. These findings suggested that the changes were due to an increase in thickness of some bones at the expense of the cavities and foramina. A further inference was that the process at fault in these −A animals was the growth of periosteal bone, whereas the endochondral growth which controlled the length and to some extent the overall size of the bones was relatively unaffected.

In 1941 (Mellanby) changes in the bone of the femur shaft were described and related to the malformation and loss of fine moulding in the cranial and vertebral bones. The shafts of the −A dogs were found to have thickened bony walls and reduced marrow spaces, and in a few cases the bones were actually larger in diameter. Their bony walls (Plate XII) were for the most part cancellous instead of compact, the amount of the cancellous relatively to the compact tissue depending on the age at which the deficiency was established, its extent, and the duration of the dieting period. The Haversian system, when present, was incompletely developed. Although the epiphyses and growing cartilage of the long bones were examined, the changes from the normal found were so small that they were not considered significant. This point, however, became of more interest because of Wolbach's report (Ludvig Hektoen Lecture, 1946) in which he discussed experiments made mainly on rats and showing the effect of various

somewhat characteristic facial appearance, producing an impression of a shorter nose.

The pelvic bones in A deficient animals have not been studied in detail, but it can be said that the effect is to make the bones thicker and less finely moulded. A finding of perhaps more importance is that the aperture and, indeed, most of the internal measurements of the pelvis, are



FIG. 29. Drawings representing the lateral view of skulls of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Note: The increased size of the malar bone (black), its growth upwards into the lower margin of the orbit, and the general thickening of the zygomatic arch (malar bone and zygomatic process of the temporal bone), in the −A dog (b)

reduced in these animals. It is unlikely that such changes could ever become as serious as the rachitic pelvic changes due to deficiency of vitamin D, and whether they are ever of practical significance is not known.

5. PERIOSTEAL AND ENDOCHONDRAL BONE DYSPLASIA

As shown above, when it was realized that bone pressure was directly or indirectly the cause of most of the nerve

lesions in −A animals, a fuller investigation into bone growth and shape was begun. Anatomical examination of the skull, vertebral column and the central nervous system revealed that the general effect of vitamin A deficiency, when reduced to its simplest terms, was a crowding of the latter into a bony box which was too small to accommodate it. The overall size of the skull and vertebral column showed only small differences quite inconsistent with the crowding of the brain. A closer examination, however, showed that there were significant alterations in the thickness of bony walls as well as in details of finer moulding. These findings suggested that the changes were due to an increase in thickness of some bones at the expense of the cavities and foramina. A further inference was that the process at fault in these −A animals was the growth of periosteal bone, whereas the endochondral growth which controlled the length and to some extent the overall size of the bones was relatively unaffected.

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PLATE XII

vitamin deficiencies and excesses on bone growth. He stated:

> "Vitamin A deficiency suppresses epiphyseal cartilage cell sequences and hence endochondral bone growth. Remodelling sequences involving concurrent resorption of bone with deposition and replacement of cancellous bone by compact bone cease to operate. Appositional growth of bone of periosteal origin continues, until inanition supervenes, at a rate in conformity to the normal growth pattern in each site. Skeletal growth as a whole ceases."

As many examinations in this laboratory of the long bones of −A dogs have never shown the drastic abnormalities at the epiphyses such as Wolbach described, it was thought advisable to refer in more detail to the slight changes which have been observed.

The determination of the differences between the epiphyseal cartilage and developing bone in +A and −A dogs is difficult, because under the experimental conditions used these differences are in many cases smaller than those which naturally occur with advancing age. Since the dogs under experiment are not of pure strain, small variations even between litter-mates of the same age are present and these tend to mask the slight changes due to deficient diet. Photomicrographs of the distal epiphyseal cartilage of the radii of three pairs of dogs are shown in Plate XIII. The animals were put on diet at the age of 6–7 weeks, one of each pair receiving a +A and the other a −A diet, and the pairs were killed after 14, 19 and 28 weeks respectively.

PLATE XII

Enlarged radiographs of the femur shafts of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Note: The compact wall in (a) and the coarse trabeculae and cancellous structure of (b).



PLATE XII

Generally speaking, therefore, this experimental work on dogs suggests that the effect of vitamin A deficiency on the growth of long bones at the epiphyses is *slight* and includes:

(1) Shortening of the calcified cartilage columns;
(2) A tendency on the part of the osteoclasts to approach nearer to the edge of the cartilage;
(3) Reduction in mitosis of the mother cell cartilage layer;
(4) An increase in bone marrow between the epiphyseal cartilage and the epiphyseal bone.

The outcome of these changes is possibly a very slight acceleration of epiphyseal closure and a similar retardation of growth. In one − A dog which was 33 weeks old, having been on the experimental diet for 26 weeks, and which lost weight during the last weeks of the experiment, the distal epiphysis of the radius had closed, whilst that of the +A control of the same age was still growing, although at a slow rate. This may, as pointed out above, have been due to an individual variation; it is of interest, however, to note that early closure of the epiphysis in rats, mice, guinea pigs, chickens and dogs has been attributed (Wolbach, 1946) to excess of vitamin A.

Since Wolbach regards the inhibitory effect of A deficiency on endochondral growth of the cranial bones as the prime cause of the degenerative change in the central nervous system, reference must be made to the results of A deficiency on these structures in the present investigation. The endochondral cartilage at the junction of the basi-occipital and sphenoid bones of +A and − A litter-mate dogs of the same age are shown in Plate XIV, c and d. As in the case of the long bones, the abnormalities in the − A animal are small and could not be responsible for the change in bone shape and size and for the subsequent pressure effects observed in the brain. It seems clear, therefore,

The differences between +A and −A dogs of the same age are slight and in no case are they as great as those between the +A dogs aged 20 and 35 weeks. The vitamin A-deficient animal killed after 14 weeks on the diet shows few abnormalities. There is apparently a slight shortening of the calcified cartilage columns, compared with those of the +A animal, and the osteoclastic activity on the bony trabeculae approaches nearer to the growing edge of the cartilage. After 19 weeks on diet the changes are rather more pronounced and there is a reduction in the number of calcified cartilage columns, with a tendency for the remaining ones to fuse laterally, but there is no change in cartilage cell maturation sequences. The third pair of dogs, after 28 weeks of the diet, appear to be approaching full growth and cartilage cell sequences, especially cell vesiculation, are reduced in both. The main osteoclastic activity is situated rather closer to the cartilage edge than in the previous pairs, and in the −A dog osteoclasts are in some places near to, and may be attacking, cells before they reach the stage of vesiculation. Even in this case, however, cartilage cell development could not be said to have ceased and in this respect bears no resemblance to the illustration of the −A dog published by Wolbach (Fig. 6, 1946). (The question of the age at which epiphyseal union takes place in the dog has not been fully investigated, but it probably varies with breed, possibly even between litter-mates of different sizes, and also, by analogy with the rat (Dawson, 1925), in different bones of the same animal.) A further change in the epiphyseal growth of the vitamin A-deficient animals, which is not clearly shown by the low power photomicrographs of Plate XIII, is a slight reduction of mitosis in the mother cell layer. It will also be seen that the cartilage and epiphyseal bone of the −A dogs are separated by rather more bone marrow than in the +A dogs. This is particularly clear in Plate XIII b and c.

that the bone abnormalities seen in the vitamin A-deficient dogs used in the present work are not primarily connected with alterations in endochondral growth. The relative importance of the effect of vitamin A deficiency on the two types of bone growth, periosteal and endochondral, is clearly seen in Plate XIV, showing photomicrographs of the distal epiphyseal cartilage of the femurs, cartilages from the base of the skulls, and portions of the basi- occipital bones of a +A and a − A dog. It will be seen that the differences in the femurs (Plate XIV, a and b) and in the cranial cartilages (Plate XIV, c and d) are insignificant compared with those in the periosteal part of the basi-occipital bones (Plate XIV, e and f). Comparisons of other bony tissues of this and other pairs of +A and − A dogs convinced the author that in this work changes in endochondral growth had not the importance claimed by some other workers, but that periosteal bone changes were of far greater consequence.

(a) *Discussion of periosteal and endochondral growth in relation to vitamin A deficiency*

Let us now consider Wolbach's experiments on rats, from which he deduced that the primary effect of vitamin A deficiency was to stop all endochondral growth, a result which clearly is at variance with the above mentioned findings in dogs. It is important to remember that, on his synthetic diet, "the rats on the deficient diets continued

PLATE XIII

Photomicrographs (× 43) of the distal epiphyseal cartilage of the radius in three litter-mate pairs of dogs of different ages.
(a), (b) and (c) +A diet.
(d), (e) and (f) −A diet.
(a) and (d) 20, (b) and (e) 25 and (c) and (f) 35 weeks of age respectively

Note: The differences between (a) and (d) (20 weeks), (b) and (e) (25 weeks) and (c) and (f) (35 weeks) are small and in no case as great as the *differences between (a) and (c), which are both* normal

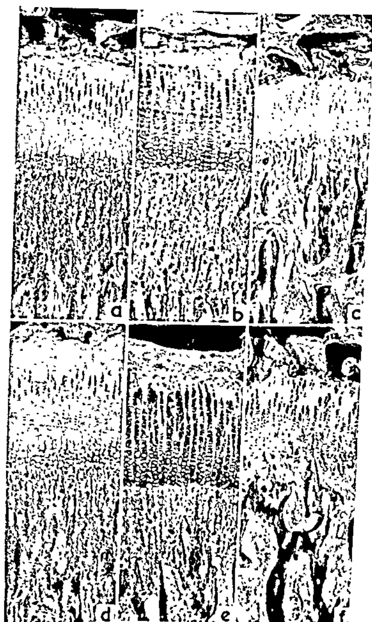


PLATE XIII

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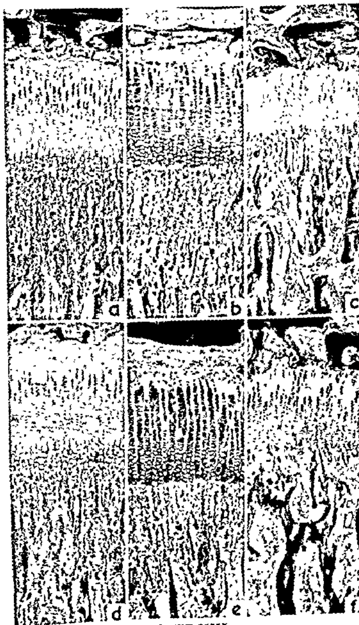


PLATE XIII

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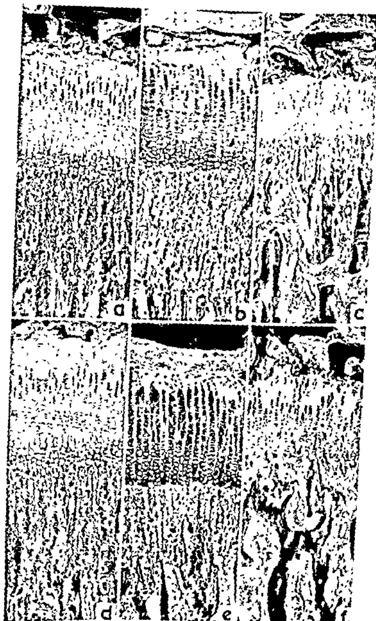


PLATE XIII

to grow at a normal rate into the sixth week of age. Stationary weight or a loss of weight was usual in the ninth week." (Wolbach and Bessey 1941) He realized that, whether vitamin A is present or not, cessation of endochondral growth is a common feature in young rats when the diet intake is diminished below a certain amount or when the absence of other specific elements brings about inanition. The difficulty in such experiments, therefore, is to decide which results are due to a true vitamin A deficiency and which to inanition and loss of weight resulting either from a deficient intake of food or from deficient supplies of specific vitamins and other chemical substances.

Repeating on a small scale Wolbach's experiments on rats, with a diet and under conditions as far as possible the same as his, which included depriving the mother of vitamin A during the latter part of the lactation period, that is to say from about the twelfth day after parturition, *similar results were obtained in regard to both the animals'* general condition and epiphyseal growth. The rats began to lose weight after about 6 weeks and died of inanition after a further 4 or 5 weeks. However, even when vitamin A supplements were added to this basal diet the growth was still subnormal, indicating one or more deficiencies other than vitamin A. Histological examination of the epiphyses showed suppression of cartilage sequences with

PLATE XIV

Photomicrographs of the distal epiphyseal cartilage of the femur (a and b) (× 39), the endochondral junction between the sphenoid and basi-occipital bones (c and d) (× 39), and a mesial sagittal section of the basi-occipital bone to show periosteal bone growth (e and f) (× 10), of two litter-mate dogs. Duration of experiment 19 weeks. Final age 25 weeks.

(a), (c) and (e) +A diet

(b), (d) and (f) −A diet.

Note: There is very little difference between (a) and (b) and probably less between (c) and (d), but there is a great difference between (e) and (f), indicating that the vitamin A deficiency at this age in dogs affects mainly the periosteal growth and only very slightly the endochondral ossification.



PLATE XIV

were put on the experimental diets when 5 weeks old and were maintained on them for 23 weeks, although the − A rat lost weight during the last 3 weeks. The illustrations of the labyrinthine capsules (Plate XV) show that the differences in periosteal bone growth in this position between *normal and deficient rats are similar to, if not identical* with, those obtained by Wolbach in his shorter experiments. The real discrepancy between the investigations is apparent when femurs and pelves of +A and − A animals (Plate XV, e-h) are examined, for here the differences in length are slight and certainly not compatible with cessation of endochondral growth. This is shown to an even greater extent in the axis vertebrae (Plate XV, c and d). As can be seen, the overall width and height of the vertebrae are similar but, as in the dog (Plate VIII), the vertebral canal is smaller in the − A rat, thus leaving less room for the central nervous system. It might be said that this was "growth in conformity to the normal pattern" (Wolbach, 1946), with a "retardation of the increase of the calibre of the canal" (Wolbach and Bessey, 1941), but the increased thickness of the dorsal spines of the vertebrae of the − A rat, which are apparent in Plate XV d, must be due to increased deposition of bone.

It will be seen, therefore, that in the rat experiments three types of result have been obtained in this laboratory:—

(1) Early suppression of endochondral ossification and relatively slight periosteal changes. This condition is produced by diets of purified substances devoid of vitamin A, if given to mother and young from about the twelfth day after parturition, so that the deficiency is established very early in life.

(2) Almost normal endochondral ossification but great overgrowth of periosteal bone, which occurs when a diet deficient in but not devoid of vitamin A is first given at

the formation of a permanent but non-growing cartilaginous disc and in some cases the formation of a bony plate across the diaphyseal face of the cartilage. If under the same experimental conditions diet 43 (see Appendix, p. 197) is substituted for that used by Wolbach, the effect on the epiphyseal growth is less severe. Animals fed on this diet continue to gain in weight until about the tenth week of age and by the twelfth week the weight usually begins to fall. Histological examinations of the distal epiphyseal cartilage of the femurs at about 11 weeks of age, when the weight is stationary, do not show a diaphyseal disc, although the impression is produced that the formation of such a disc may be beginning. Diet 43 includes oatmeal and therefore contains a trace of carotene, but nevertheless all bone growth stops prematurely and the epiphyses show, as would be expected, many of the changes associated with cessation of growth and with $-A$ diets composed of pure substances.

It may be asked how rats would react to diets which did not produce vitamin A deficiency so early in life, and from which better and more sustained growth was therefore obtained. Rats maintained on a normal stock diet, which includes bread, milk and cod-liver oil, up to the age of 3–5 weeks and then put on the vitamin A-deficient diet 43, show bone changes similar to those seen in dogs. The long bones are almost equal in length to those of the $+A$ rats, but are coarser in shape and less finely moulded, indicating again that, whereas the vitamin deficiency has little effect on the endochondral growth, as judged by the overall size of the bones, it results in abnormal growth of the periosteal bone. In order to demonstrate these facts, photographs of some rat bones are shown in Plate XV. These include the labyrinthine capsules, axis vertebrae, femurs and portions of the pelves of two litter-mates which

the age of 3–5 weeks to rats with a good vitamin A reserve; the deficiency therefore begins at a later stage. (This result is similar to that obtained in the dog experiments.)

(3) An intermediate condition in which cessation of growth does not take place until the young rats are about 12 weeks old, and there is then both interference with the endochondral ossification and definite slight overgrowth of the periosteal bone. This occurs when diet 43 is given to rats which have been allowed to run out of vitamin A at an earlier age by removing the vitamin from the mother's diet during lactation.

Although Wolbach gives no details of the dietetic regime of his – A dog, it can probably be assumed from the general text that the conditions were similar to those for the rat and that the – A dog lost weight and was suffering from inanition before the experiment ended. The many dogs used in the present investigation were not given the deficient diet until they were 6–8 weeks old and, since this diet was composed of natural foodstuffs, it was not completely devoid of vitamin A and/or carotene. The dogs whose long bones were sectioned, with one exception (p. 131), neither lost weight nor suffered from inanition; nor did they show any but the slightest changes in epiphyseal cartilage (plate XIII). Had these animals been subjected to an earlier and more severe deprival of vitamin A (and possibly of other

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reduced size of the internal auditory meatus (IAM) and the fossa of the parafloccules (FP) of the temporal bone in (b) when compared with (a). The foramen lacerum appears to be greatly reduced in size due to part of the foramen being underneath the swollen petrous ridge.

(2) The overall size of the 2nd cervical vertebrae is similar in (c) and (d), but in (d) the spinal canal is smaller and the dorsal spine thicker.

(3) Although there is some thickening and loss of finer moulding at the lower end of the femur shaft in (f), there is no great difference in length between (e) and (f).

(4) The overall size of the pelvic bone is approximately equal in (g) and (h)

These results show that under certain conditions, (p. 136) deficiency of vitamin A does not stop epiphyseal growth in rats, but does affect periosteal bone growth.

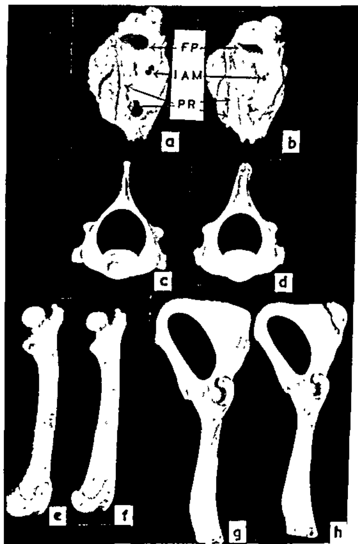


PLATE XV

Photographs of various bones from two litter-mate rats. Age when first given the experimental diet 5 weeks. Duration of experiment 23 weeks.

(a), (c), (e) and (g) +A diet 43.

(b), (d), (f) and (h) −A diet 43.

Note: (1) Great overgrowth of the petrous ridge (PR) and

tion of efferent and afferent neurones can be explained by the following:

(a) Differences in the degree of bone pressure to which they are subjected;

(b) Direct action of bone pressure on sensory ganglia;

(c) Variations in susceptibility of nerves to pressure.

(2) Malformed bone of the skull and vertebrae probably accounts for most of the differences in the amount and in the type of nerve lesions found in the central nervous system. The abundant degeneration in the second ascending neurones of the ventral and dorsal spino-cerebellar tracts still, however, is difficult to understand, although it may be transneuronal in origin.

(3) Differences in susceptibility of adult and growing dogs, respectively, to vitamin A deficiency seem to be satisfactorily explained on the basis that bone malformation is a primary lesion.

(4) Bones unrelated to the central nervous system, such as the pelvis and malar bones, are malformed.

(5) Under the conditions of this investigation, changes in the growth of periosteal bone are of far greater importance than the inhibitory changes affecting endochondral growth in bringing about the gross shape and absence of moulding in cranial and vertebral bones which are responsible for the destructive changes in the nervous system.

Evidence has been given which suggests that in the rat the more severe the vitamin A deficiency and the earlier in life its onset, the more likely is interference with endochondral growth associated with inanition to occur: periosteal (appositional) bone growth is also abnormal even at early stages and remains a prominent feature at later ages when the endochondral bone growth is hardly affected by A deficiency.

unknown substances, as in the rat experiments), they might have lost appetite and suffered from inanition, and, if so, they might have shown the same cessation of bone growth at the epiphyses as the rats described by Wolbach and in section (1) above. Cessation of growth of the long bones seems to have occurred in the dog described by him (Fig. 6, 1940), and it would be interesting to know whether this dog did in fact lose weight before death.

It would appear, therefore, that probably the main discrepancy between the results of the present work on dogs and rats and those of Wolbach on rats can be explained by differences in the age at which vitamin A reserves of the body are exhausted and in the degree of inanition which the animals develop. The earlier in life this stage is reached, the more likely is it for inanition to develop and for growth at the epiphyses to be stopped, although apparently abnormal periosteal growth also occurs at most stages. It may be that in rats developing nerve degeneration at early ages the periosteal bone abnormalities develop before the state of inanition and subsequent cessation of bone growth occur. It is difficult to believe that periosteal bone continues to be laid down, sometimes to an excessive degree, at a time when all endochondral growth has ceased. It is, however, clear that the one abnormal change in bone produced by a vitamin A deficiency, whatever the age of the animals, extending even to the adult, is that affecting the periosteal bone.

5. Summary

In this chapter a number of problems that have arisen in the course of the work are discussed and evidence is given to show that in animals brought up on diets deficient in vitamin A,

(1) The differences in relative susceptibility to degenera-

pace with the increasing length. This remoulding is carried out by bone cells, osteoclasts and osteoblasts, of the endosteum and periosteum and the bone laid down in this way is sometimes referred to as appositional growth. Obviously a carefully controlled balance must be maintained between the activities of these two types of cell, and anything which alters their position or relative activity will result in abnormally shaped bones.

Applying these ideas to the study of the appositional bone of A-deficient animals, there are two actions to be considered. There is in some places an actual laying down of superfluous bone due to excessive osteoblastic activity. This is clear in the case, for instance, of the internal auditory meatus (Plate V, a and b), for, in the − A animals new bone may almost occlude the meatus. Similarly, the folding of the bone around the fossa of the parafloccculus, the second, fifth and other foramina, indicates excessive laying down of new bone.

All the extra bone found in − A animals, however, cannot be accounted for so simply. In some cases it is evident that the mechanism at fault is not so much bone overgrowth as absence or diminution of absorption of older formed bone. In Fig. 20, for instance, it will be noticed that the distance from the outer side of the supra-occipital to the lower margin of the basi-occipital, SB, S′B′, is practically the same in +A and −A dogs. The difference is not in the outside but the inside measurement, i.e. from the lower margin of the supra-occipital to the upper margin of the basi-occipital, PO, P′O′. The same applies to the smaller spinal canal of the vertebrae in the − A animals (Plates VIII and XV). This can be explained on the hypothesis that, while the vertebrae grow at least as much as those of +A dogs on the outer margins, there is a diminished and irregular absorption of the bone lining

Chapter VIII

CELL ACTIVITY IN GROWTH AND MODELLING OF BONE

The discovery of the effect of vitamin A on bone shape during the period of growth of the animal was obviously a matter of great interest. In the first place, it seemed to afford a satisfactory but completely unexpected explanation of a phenomenon, namely nerve degeneration in a deficiency, which had been pursued for many years. More important, however, it opened up a new chapter in bone physiology and pathology. It seemed possible that insight into processes normally controlling the growth and shape of bones might be obtained by further study of this phenomenon. It was particularly desirable to see how the cells responsible for endosteal and periosteal growth were influenced by vitamin A deficiency.

1. OSTEOBLASTS AND OSTEOCLASTS

Most bones are first laid down in cartilage and it is endochondral growth that largely determines their increase in length and, to a varying degree, their actual size. Pari passu with the endochondral growth, periosteal bone is laid down and it is this activity which is mainly responsible for the moulding and finer shape of the bone. Taking the basi-occipital bone as an example, it is evident that endochondral growth at its anterior end will result in lengthening of the base of the posterior fossa, so that the growing brain stem can be suitably accommodated. The shape of this bone is, however, delicately adjusted to that of the lower surface of the pons and medulla and, in order to maintain this adjustment, remoulding, both of the new and of the older, earlier formed bone, must keep

pace with the increasing length. This remoulding is carried out by bone cells, osteoclasts and osteoblasts, of the endosteum and periosteum and the bone laid down in this way is sometimes referred to as appositional growth. Obviously a carefully controlled balance must be maintained between the activities of these two types of cell, and anything which alters their position or relative activity will result in abnormally shaped bones.

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PLATE XVI

Photomicrographs (× 125) of sagittal sections of the inner table of the basi-occipital bones of two litter-mate dogs to show reversal of osteoclastic and osteoblastic activity on surfaces 1 and 2. Duration of experiment 19 weeks. Final age 25 weeks.

(a) +A diet. Surface 1, many active osteoclasts, no active osteo-

the vertebral canal. The central nervous system, its membranous coverings and the fluid which surrounds it are thus compressed by the bones and the nervous tissue is ultimately damaged. If these suggestions are correct, it ought to be possible to demonstrate their truth, because the function of the osteoclasts is to remove bone and therefore a difference would be expected between the number and activity of osteoclasts on the inner side of these bones, i.e. the side adjacent to the central nervous system, in +A and − A dogs. A full histological examination of the occipital and vertebral bones was made in many of the experimental animals and these showed the expected differences.

Plate XVI shows photomicrographs of corresponding portions of the internal surface of basi-occipital bones of +A and − A animals. It will be seen that, whereas in the +A animal there were abundant active osteoclasts on the surface of the bone nearest the brain and directly in contact with the bone, there were none in this position in the section from the − A animal. This point is shown more clearly in the semi-diagrammatic Fig. 30 (of which Plate XVI is a part). The section of bone in each case was drawn under camera lucida, but the osteoclasts seen in the histological preparation are shown as black dots. Here again it will be seen that the osteoclasts in some places are absent from, and in other parts greatly reduced on, the brain surface of the bone in the − A animal, but there are far more of these cells on the marrow surface than in the +A animal. In other words, the active osteoclasts seem to have been reversed in position. A similar change has been observed in the vertebral bones of −A animals, an example of which is given in Fig. 31, which represents corresponding

blasts. Surface 2, many active osteoblasts, no active osteoclasts (See Fig. 34 for plan)

(b) −A diet Surface 1, many active osteoblasts, no active osteoclasts Surface 2, many active osteoclasts, no active osteoblasts

halves of vertebrae of +A and − A dogs, and which is also of a semi-diagrammatic nature. Again it will be seen that active osteoclasts are few on the internal surface of the bony canal in the − A animal, whereas they are abundant in the corresponding position in the +A animal. On the other hand, there are more active osteoclasts on the marrow

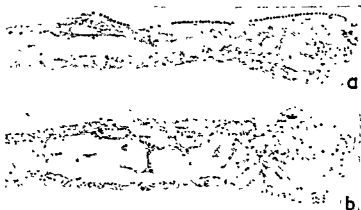


FIG. 30 Semi-diagrammatic drawings of the basi-occipital bones of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet
Osteoclasts are indicated by black dots.
Note: (1) Thickening of bone in (b) as compared with (a).
(2) In (a) there is a large number of osteoclasts on the surface of the bone adjacent to the brain (upper surface in diagram). In (b) they are absent from this region, but are abundant on the marrow surface of the same portion of bone. There seems to have been a reversal of the position of activity of the osteoclasts.

surface of the bone in the − A than in the +A. As in the basi-occipital, there is again a reversal of position of the active cells. Thus we see that in growing bones surrounding the central nervous system the normal mechanism is for absorption to predominate on the surfaces adjacent to the brain and spinal cord whilst deposition predominates on the outer surfaces. This will have the effect of increasing

the size both of the cranial and of the vertebral cavities, thereby providing part of the extra space required by the growing central nervous system. This mechanism breaks down in − A animals, since the osteoclasis ceases in some positions where it normally occurs, and is found on other surfaces where such activity is abnormal. This explanation of the breakdown of the physiological growth fits the facts of the investigation as regards these bones, but it is not easy to understand. While lack of absorption of bone in the right place is the main feature in the occipital and vertebral bones of − A dogs and is undoubtedly responsible *for many of the destructive changes of the nervous system* described, it is not the only abnormality. There are obvious changes also in osteoblastic activity even in these bones. In Plate XVI, for instance, reversal of position of activity of osteoblasts as well as osteoclasts can be seen.

Thus it appeared at this stage of the work that the process by which bones became abnormal in shape was a reversal in the activity of the predominant type of bone cell at various surfaces. This, however, did not prove to be completely true, for in some situations, notably the *surface of the labyrinthine capsule around, but not within,* the internal auditory meatus, there was no reversal of cell type. Here is a structure which is nearly fully formed at the time the deficient diet is begun and yet the deficiency of vitamin A starts great osteoblastic activity which thickens the labyrinthine capsule so that it compresses the brain stem. In other situations, for instance around the foramen lacerum, although osteoclastic activity is reduced and the foramen therefore less in diameter than the normal, complete loss of osteoclasis has not been observed. Since the internal carotid artery passes through this foramen, stenosis in this position would be followed by the rapid death of the animal and it may be that some special mechanism protects such structures. An intermedi-

halves of vertebrae of +A and − A dogs, and which is also of a semi-diagrammatic nature. Again it will be seen that active osteoclasts are few on the internal surface of the bony canal in the − A animal, whereas they are abundant in the corresponding position in the +A animal. On the other hand, there are more active osteoclasts on the marrow

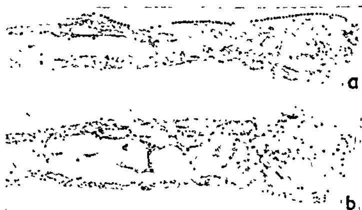


FIG 30. Semi-diagrammatic drawings of the basi-occipital bones of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Osteoclasts are indicated by black dots.
Note: (1) Thickening of bone in (b) as compared with (a).
(2) In (a) there is a large number of osteoclasts on the surface of the bone adjacent to the brain (upper surface in diagram). In (b) they are absent from this region, but are abundant on the marrow surface of the same portion of bone. There seems to have been a reversal of the position of activity of the osteoclasts.

surface of the bone in the − A than in the +A. As in the basi-occipital, there is again a reversal of position of the active cells. Thus we see that in growing bones surrounding the central nervous system the normal mechanism is for absorption to predominate on the surfaces adjacent to the brain and spinal cord whilst deposition predominates on the outer surfaces. This will have the effect of increasing

ate type of change is seen in the bone around the dorsal root ganglia, on the surface of which there is normally much osteoclasis. In many −A dogs only reduced osteoclastic activity is seen in this position (Fig. 31), but in severe cases all active osteoclasts may disappear. Thus we see that the effect on bone cell activity of removing vitamin A from the body is to cause reactions which are determined to some extent by the normal. For instance, on a surface where there is normally no cell activity, a strong osteoblastic one may develop. A surface on which there is little absorption may now show deposition of bone, and one on which there is a strong osteoclastic activity may show reduced activity, or in severe cases none at all.

Obviously these variations, often in different parts of the same bones, may, when fully understood, add to our knowledge of bone growth. Let us first examine the question of osteoblastic and osteoclastic activity on bone surfaces, apart from any influences affecting them. Early in the work it seemed difficult to understand how osteoblasts and osteoclasts could be placed on opposite surfaces of a plate of bone and how at a later stage these same surfaces could be covered by the other type of cell. The idea that bone cells actually changed sides according as to whether vitamin A was present or not was difficult to accept. It is now evident that there are either active or inactive cells of both

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FIG. 31. Semi-diagrammatic drawings representing the lateral portions of comparable vertebrae of two litter-mate dogs of the same age.
(a) +A diet.
(b) −A diet.
Osteoclasts are indicated by black dots.
Note: (1) Enlargement of bones in (b) as compared with (a).
(2) In (a) the osteoclasts on the surface of the bone adjacent to the spinal cord are numerous. In (b) relatively few osteoclasts are found in this position. On the marrow side of this same portion of bone, however, they are more numerous than in the corresponding position in (a). There seems, therefore, to have been a reversal of the position of activity in the −A animal.



Fig. 31.

on surfaces where, under other circumstances, they are apparently roused to activity and in the process become rounder and more clearly multinuclear. Osteoclasts of both forms are seen in Plate XVII, c–f. Occasionally active osteoclasts appear in great numbers on some bone surfaces where few if any inactive osteoclasts can be distinguished in the normal. In such cases it might be that these active osteoclasts were either transferred to the surface or possibly formed there from other precursors.

As regards the conditions affecting the bone cell activity, it was thought that some insight might be gained from an *examination of relative rates of growth of various bones* at different ages. Because of the rapidity with which the abnormalities described above can be produced in young growing animals as compared with the slow production of bone changes in adult animals, it might be expected that bones normally growing most at a given age would, in A-deficient animals, show the greatest dysplasia, especially if they were in close proximity to the nervous system so that the most rapid growth had to be accompanied by a vigorous moulding system to allow perfect adjustment between growth of nervous tissue and of the surrounding bone. If under such conditions the bone cells worked in a wrong direction, the shape of the bone would not only be grossly abnormal but the ill effects on the associated growing nervous tissue would be great. It was shown in Chapters V and VII that in the cranial bones abnormal growth in A deficiency was greatest in the posterior fossa. Therefore, if this cavity were shown to enlarge at a greater rate than the anterior or middle fossa, and if this quicker growth occurred between the 6th week and the 7th month of life (i.e. the usual experimental period), the idea of a relationship between rate of growth and dysplasia would be supported. This supposed quicker growth of the bones *surrounding the posterior fossa* would be expected to

types present on most bone surfaces in the period of rapid growth, and that different circumstances, for example the presence or absence of vitamin A, activate one or other type



PLATE XVII

Photomicrographs of sections through basi-occipital bones to show inactive and active osteoblasts and osteoclasts.

(a) Inactive osteoblasts Cells flattened on bone surface (× 325)

(b) Active osteoblasts Cells cubical on bone surface (× 325).

(c) Inactive osteoclasts. Cells lying flattened between bone surface and periosteum; very little evidence of bone absorption (× 228).

(d) Active osteoclasts Multinucleated cells prominent between bone surface and periosteum; note lacunae due to absorption (× 228).

(e) One inactive osteoclast (× 650).

(f) One active osteoclast (× 650).

of cell. Flattened inactive (Plate XVII a) and enlarged active osteoblasts (Plate XVII b) are found on many bone spicules. Inactive flattened osteoclasts also can be seen

are mainly filled with fatty marrow. Although the bone usually appears to be an immature lamellar type lacking the Haversian system, the minute structure is not far removed from the normal. This delay in the conversion of the lamellar to the Haversian type is often prominently seen in the long bones of − A animals. There is no osteoid tissue, as in rachitic bones, nor is there any inflammatory reaction. The presence of osteoid tissue, of course, would not be expected as the diets are rich in vitamin D. Chemical analysis shows that the actual amount of calcium in corresponding bones of +A and − A animals is roughly the same. Taking an average amount of calcium in the femur shaft per 1000 grms. of body weight in groups of ten +A and ten − A animals, it was found that the +A group had 0.131 grms., while the corresponding figure for the − A group was 0.134 grms. It seemed possible that the cancellous overgrowth in − A animals indicated an effort by bone osteoclasts to supply the necessary salts to harden the additional new bone laid down by the active osteoblasts. It did not seem probable that phosphorus was a limiting factor, since the diets, based on experience in the laboratory, were adequate in this respect. On the other hand, the calcium of the diet was known to be low for rapid optimal calcification, and it seemed possible that if abundant calcium salts were supplied, bone overgrowth would be prevented. Experiments were therefore made to see what would happen if the calcium intake were increased in both +A and −A dogs. The results of one such experiment on the structure of the cribriform plate is shown in Fig. 32. It will be seen that the effect of adding extra calcium in the form of calcium carbonate to the diet has been to make the cribriform plate in both dogs more compact and less cancellous. On the other hand, the plate of the −A dog is still much thicker than that of the corresponding +A dog. Other bones showed similar results.

coincide with an increased growth of the cerebellum and brain stem when compared with the brain as a whole, which, in fact, seems to be the case. Taking the average weight of these parts of the brain to the total brain weight at different ages in a number of normal dogs, it was found that the cerebellum, pons and medulla formed about 13 per cent of the whole brain weight at 6 weeks of age, 15 per cent at 22 weeks, and over 17 per cent at 34 weeks. There is, therefore, evidence of a relatively greater increase in size of the hind brain during the experimental period and, it can be presumed, of its bony covering, leading to an increased need for remodelling sequences.

Although there may be some truth in this relationship, it can only be a partial truth, for, as previously pointed out, one of the most dramatic and harmful changes in bone shape produced by vitamin A deficiency is that experienced by the surface of the labyrinthine capsule around the internal auditory meatus, which is nearly fully formed before the usual experimental feeding period commences. The bone hyperplasia at this point resulting from lack of vitamin A, therefore, has no obvious relation to the normal growth at this period of life. As was seen in the variation of the effect of A deficiency on bone cells at different points, the whole question of bone growth and moulding and their coördination is so complicated that a simple answer to this part of the problem cannot be expected until it has been studied further. In Chapter IX this question is more fully discussed, especially the changes in bone cell activity at different places brought about by vitamin A deficiency and the changes which follow restoration of the vitamin to the body.

2. Chemical Considerations

It was observed previously, that the overgrown bone is usually of cancellous structure and that the interstices

ence is particularly great in animals which have been sufficiently long on the diets to allow large bone changes in the −A dogs. The significance of the high fat content is not known.

3. Summary

It has been shown that in dogs brought up on diets deficient in vitamin A,

(1) The position and intensity of activity of osteoclasts and osteoblasts especially in growing bones are often altered. There is usually partial, or sometimes complete, reversal of the normal cell activity, so that appositional bone may be laid down in places in which absorption should predominate and remain in other places from which normally it should be absorbed.

(2) The findings as regards osteoclastic and osteoblastic activity explain the nature of the bone malformation, namely, coarseness in shape and especially the lack of remoulding. Since, however, different bones show variations in intensity of the reactions of the osteoclasts and osteoblasts to −A conditions, there are clearly other factors at work whose nature and mode of action in controlling bone growth and shape are unknown.

(3) Developing bones, in which most growth and remodelling are normally found, usually show the greatest malformation, but excessive bone formation may also be found in areas where the normal growth has for the most part ceased.

(4) The total amount of calcium salts in the femur shaft and other bones is about the same as in the corresponding bones of the control animal, in spite of the larger bulk.

(5) Although extra calcium in the diet may modify the type of bone, it does not prevent the production of abnormal bone shape.

(6) The bones often contain more fat than those of normal dogs.

In other words, calcium is not the controlling factor as regards bone hypertrophy, but a larger supply than that in the basal diet of many of the experiments does influence the bone structure and makes the bone more compact.

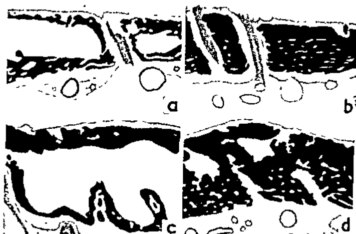


FIG. 32. Drawings representing sections of the cribriform plate of four litter-mate dogs of the same age.
(a) +A diet.
(b) +A diet and extra Ca (2 grms. $CaCO_3$ daily).
(c) −A diet.
(d) −A diet but with extra Ca (2 grms. $CaCO_3$ daily).
Note: A deficiency of vitamin A (with or without extra Ca) results in thickening of the cribriform plate. The addition of Ca does not, therefore, prevent bone overgrowth. Addition of Ca with or without vitamin A increases the compact and reduces the cancellous bone. In the +A animals (a and b) there is ample space for the bundles of nerve fibres passing through the foramina of the cribriform plates In the −A animals (c and d), however, the foramina are reduced in diameter and the nerves are squeezed.

A second point of interest is the relatively high fat content of the bones of some of these −A dogs as compared with that of similar bones of +A litter-mates. For instance, the average fat content of the femur shaft per 1000 grms. of body weight in groups of ten +A and ten −A animals was 0.173 grms. and 0.566 grms. respectively. This differ-

recovery continues, but at a somewhat slower rate. When a puppy has been maintained on a vitamin A-deficient diet for as long as 5 to 6 months, the addition of the vitamin will bring about a great improvement in its condition, but even after 7 or more months of this therapy it will still be abnormal. This indicates that not only have some central and peripheral nerves been destroyed during the period of depletion but that all the lost functions and defects have not been compensated for by the opening up of other nerve paths.

The effect of the addition of the vitamin to A-deficient animals can sometimes be easily followed by examining the eyes. For instance, the sight, which may have been impaired during the period of deficiency, is often noticeably improved within a few days of the change in diet and, although suitable tests for vision have not been designed, the ophthalmoscopic appearance of the disc gives some indication of the course of the improvement. In the case of one particular dog in which papilloedema (swelling of +2D) had been diagnosed after 16 weeks on the deficient diet, only 8 weeks of vitamin A therapy were needed to correct this condition. (In other cases the time needed for recovery has varied between 2 and 8 weeks.) During this same period the pallor and the uneven, feathery outline of the disc disappeared and the ophthalmoscopic appearance returned to normal. The eyes of other species of animals also respond to vitamin A therapy after a period of deficiency. As was stated elsewhere (p. 51), early xerophthalmia in rabbits may clear up in a few days after the addition of the vitamin, and improvements have also been recorded in rats and ferrets.

It may be objected that some of these changes due to A deficiency, such as xerophthalmia, are not dependent on the nerve changes described above (see Chapter III) and that their cure on readministration of vitamin A does not

Chapter IX

RECOVERY CHANGES ON RESTORATION OF VITAMIN A TO THE DIET

When the effects of vitamin A deficiency on bone cells and so on the shape of the bones of growing animals were described in the Croonian Lecture of 1943, the obvious question was posed whether these effects would be reversed when vitamin A was again added to the defective diet (Mellanby 1944). It was apparent at the time that, whatever reverse effect might follow the re-introduction of vitamin A into the diet, complete recovery from the ataxia and other signs of A deficiency could not be expected in all animals, since some of the peripheral nerves, such as the optic and auditory, and the tracts in the central nervous system could not recover from the destructive changes. It was known (Mellanby 1938) that recovery changes from A deficiency—often a great degree of recovery—followed the administration of vitamin A, the recovery being most complete in early and slight cases.

1. Recovery in Animal Behaviour

Recovery changes can be brought about in these A-deficient animals by adding the vitamin either as a crude liver extract or in a purified form as vitamin A acetate or carotene. There is a rapid effect on the general behaviour of such animals, including a great increase in activity which in itself may at first have the effect of making incoördination of movement appear worse, but obvious improvement in gait and balance generally follows quickly, in perhaps 7 to 10 days, if the period of A deficiency has been relatively short. If the deficiency is of longer duration, there may be an apparent lag after the initial response, during which

that the degree of recovery following the administration of vitamin A would have a similar distribution. This proved to be the case and the description of macroscopic bone changes seen when vitamin A was added to the diet of a deficient animal are confined to the posterior part of the skull. These changes in shape are shown in Fig. 33a, b and

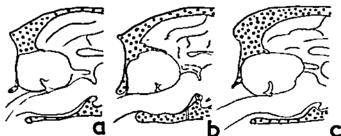


FIG. 33. Diagrams of mesial sagittal sections of the posterior fossa of three litter-mate dogs.
(a) +A diet (27 weeks).
(b) −A diet (27 weeks).
(c) −A diet for 27 weeks and then +A diet for a further 28 weeks

Note: (1) The compression of the brain stem and the herniation of the cerebellum into the foramen magnum in (b) as compared with (a) and its return to normal in (c).
(2) The thickening of the basi-occipital bone in (b) and its return almost to normal in (c).
(3) The supra-occipital and parietal bones however in (c) remain enlarged (see text). Animal (c) was considerably older than (a) and (b) and as was pointed out in the text this increase in age may explain the thickness of these bones.

c, which represent the appearance of the posterior portion of the skull after mesial sagittal section.

All three puppies were first given the experimental diets when 8 weeks old. One animal (a) was given a daily supplement of vitamin A throughout the experimental period and was killed after 27 weeks. The other two animals (b) and (c), were given the vitamin A-deficient diet only for 27 weeks. Animal (b) was killed at this stage, but (c) was then given a daily supplement of vitamin A for a

indicate that changes in shape of bone are reversible by the same means. It will, however, be agreed that the papilloedema of the disc is directly related to changes in intracranial pressure and that these in turn depend on abnormality of bone growth.

The defect in A-deficient animals that is most apt to remain even after vitamin A therapy is deafness. As previously shown, destruction of the auditory branch of the VIIIth nerve is one of the earlier effects of vitamin A deficiency and once these nerves have degenerated no recovery can take place.

It will be seen, therefore, that there was good a priori evidence that the addition of vitamin A to the diet of an animal suffering from a deficiency of this substance brought about a reversal of those pathological changes which were primary to most of the other developments.

2. Recovery in Bone Shapes

If, as the foregoing evidence indicated, bone changes held this primary position, it would be expected that, following the readministration of vitamin A, they would be completely or partially reversed; and also that the bone cell processes, which had been shown to be responsible for the abnormality, would show a return towards the normal position of activity. It is now proposed to give evidence on these points. First the changes in the shape of the skull bones due to A deficiency, and the reversal towards the normal following vitamin A therapy, will be discussed, the whole being considered in relation to the space occupied by the central nervous system. Later the subject will be dealt with in terms of bone cell activity.

It was seen in Chapters V and VII that the greatest changes in bone shape were found around the posterior fossa, the bones surrounding the middle and anterior fossa being relatively little affected. Therefore it was expected

unfortunate that no +A litter-mate control of this age (63 weeks) was available and that comparison had to be made with the +A animal aged only 35 weeks, for it seems from examinations of other dogs that the size of the supra-occipital, especially in the region of the occiput, and the parietal bones, may normally become thicker between the ages of 35 and 63 weeks. Even if some of the extra thickness is due to normal growth, the supra-occipital and parietal bones have not been remoulded to the same extent as the basi-occipital bone. It is clear, therefore, that the skull of the "recovery" animal (Fig. 33c) has not returned to the condition of the normal animal (a), but it is certainly more like (a) than is that of the −A animal (b), especially in regard to the space for the cerebellum and medulla.

If the cerebellum and brain stem are now removed from the posterior fossae of these three skulls, a further comparison can be made. The labyrinthine capsule, which has been shown to undergo such large changes in the −A animal has receded in the "recovery" animal (c) as compared with the − A animal, but here again the return is not complete, the capsule remaining very slightly convex instead of being slightly concave. The petrous ridge also is a little bulbous, but in both regions the improvement as compared with the A-deficient animal is great. To sum up, the evidence suggests that the dysplasia of the bones of the posterior fossa produced by vitamin A deficiency is mainly reversible, but it does not seem that recovery is complete in all cases and a certain amount of malformation probably persists in some bones.

3. Recovery Changes in Bone Cells

Having described briefly the naked-eye change in some of the skull bones after the restoration of vitamin A to the diet of A-deficient animals, it may be of interest to note the changes observed when the bones are examined under

further 28 weeks. Thus the condition of the bones in (b) should indicate the approximate degree of bone change present in (c) at the time the vitamin A was first added to the A-deficient diet of (c).

Comparing Fig. 33a (+A) with 33b (−A), it is obvious that a condition similar to that previously described (p. 76) has been produced by A deficiency. The basi- and supra-occipital bones are thickened at the expense of the posterior fossa, so that the brain stem and cerebellum are compressed and the cerebellum is forced posteriorly into the reduced aperture of the foramen magnum. The tentorium cerebelli is calcified, and the parietal bone thickened, and there is considerable enlargement of the posterior clinoid process. If Fig. 33c is now examined, it is at once clear that some bones have been reduced in size in the recovery period: the basi-occipital bone, for instance, has returned to approximately the same size as in the +A animal (a), the posterior clinoid process is smaller than in either (a) or (b) and there is no calcification of the tentorium cerebelli. The effect of these changes on the posterior fossa, and therefore on the shape of the hind brain, is great, for the cerebellum has practically regained its normal shape, the brain stem is no longer compressed and forced into an unnatural shape, as in (b), and the subarachnoid cisternae ventral to the brain stem have reappeared. The diameter of the foramen magnum has increased and is in (c) greater than in the +A dog, showing that full growth had not been reached when the +A experiment (a) was terminated. The same point also arises when the supra-occipital and parietal bones are examined, for these bones are larger in (c), the "recovery" animal, than in either (a) or (b). It was at first thought that this indicated an absence of any "recovery" change, for it did not seem possible that these bones could have been larger when the vitamin was added than they now were. It was

table. Surface 1 is adjacent to the central nervous system, 2 and 3 are adjacent to the marrow and 4 is the external surface of the bone.

On page 143 it was shown that a −A diet produced a thick cancellous basi-occipital bone, the abnormal thickness of which was caused largely by lack of absorption of

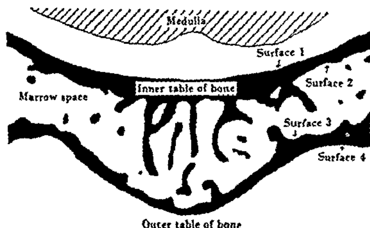


FIG 34 *Diagram of the general structure of the basi-occipital bone.* Surfaces have been numbered to simplify description in text. Surface 1 is the bone surface adjacent to the brain stem; it is separated from the nervous tissue by closely attached periosteum and other membranes
Surface 2 is the marrow surface of the same inner table of bone.
Surface 3 is the marrow surface of the outer table of bone.
Surface 4 is the outer surface of the outer table of bone.

surface 1, indeed, actual desposition of additional bone on this surface (Plate XVIb) could be seen at certain early stages of growth on these deficient diets. It appeared that absorption was greatly reduced or had even ceased because the osteoclasts, normally active and abundant in this position during growth, gave way to active osteoblasts. Conversely, on surface 2 osteoblastic activity had ceased and been succeeded by osteoclastic activity. This is shown

the microscope. Descriptions will therefore now be given of the histological appearance of several bones in the region of the central nervous systems of litter-mate puppies of (1) normal growth, as found when vitamin A is included in the diet throughout life, (2) abnormal growth due to A deficiency, and (3) bone showing the effect of the addition of the vitamin to the diet after a period of A deficiency.

The addition of vitamin A to the diet of an A-deficient animal during growth generally brings about a return of osteoclastic and osteoblastic activity to those surfaces of bone where it is normally found during the active period of growth. This reaction is often of an intense nature and its object is apparently to restore as far as possible the normal shape and quality of the dysplastic bones.

The detailed study of these phenomena made it clear that the mode of action of vitamin A in normal bone growth cannot be interpreted as easily as was at first thought. In many cases the restorative action of the vitamin, which is an exaggeration of the normal, helped to elucidate the normal method of growth and showed the different means (e.g. variation in numbers, in intensity of activity and in position) whereby osteoclasts and osteoblasts bring about the type of growth necessary in a particular bone or part of a bone.

Four regions of the skull will now be described from this point of view, viz., the basi-occipital bone, the sphenoid bone and the IInd nerve foramen, the petrous ridge and the Vth nerve foramen, and the labyrinthine capsule in the neighbourhood of the internal auditory meatus.

(a) *Basi-occipital bone*

To simplify the description of this bone, reference will be made where possible to surfaces 1–4 in Fig. 31. Surfaces 1 and 2 limit the inner table of bone, 3 and 4 the outer

table. Surface 1 is adjacent to the central nervous system, 2 and 3 are adjacent to the marrow and 4 is the external surface of the bone.

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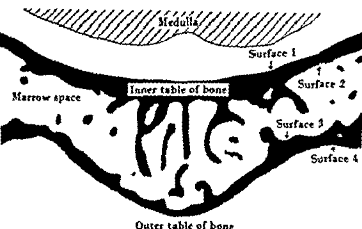


FIG. 31 Diagram of the general structure of the basi-occipital bone. Surfaces have been numbered to simplify description in text.
Surface 1 is the bone surface adjacent to the brain stem; it is separated from the nervous tissue by closely attached periosteum and other membranes.
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in photomicrographs of mesial sagittal sections of the basi-occipital bone (Plate XVI). Vitamin A deficiency caused but little change on surfaces 3 and 4, so that the outer table of bone continued to grow normally and therefore to withdraw from the inner table, with the consequent enlargement of the marrow cavity and thickening of the bone.

The above facts apply to dogs killed when 20–30 weeks old, which, judged by their behaviour during life, had been depleted of vitamin A stores for 8 or more weeks. In older animals the rate of growth of the basi-occipital bone diminishes and the differences between the number and activity of osteoclasts and osteoblasts on surfaces 1 and 2 in +A and −A dogs, although still evident, were not as obvious as in the younger animals (Plate XVIII, a and b).

Drawings of coronal sections of the central portion of the basi-occipital bone of the older +A and −A littermates, which were 33 weeks old at death, can be seen in Fig. 35. In the +A dog (Fig. 35a), which had 5000 i.u. vitamin A daily, the inner and outer tables of bone have fused to form a compact structure and the marrow cavity has been eliminated. In the −A dog (Fig. 35b) the bone is cancellous, with a large marrow space, and surface 1 is almost flat as compared with that of the +A animal, which is modelled to the natural shape of the brain stem. It will be seen that on this surface in the +A dog there are still abundant osteoclasts which are more numerous in the centre than at the sides, a fact which may account for the hollowing near the mid-line. In the −A dog, on the other hand, there are osteoblasts at work, but no active osteoclasts. Thus, so far as surface 1 is concerned, the absence of vitamin A, as in the younger animal previously described, has reversed the type of bone cell at work. Surface 2, however, of the older −A dog (Plate XVIII) is different from that of the younger animal (Plate XVI) in that there

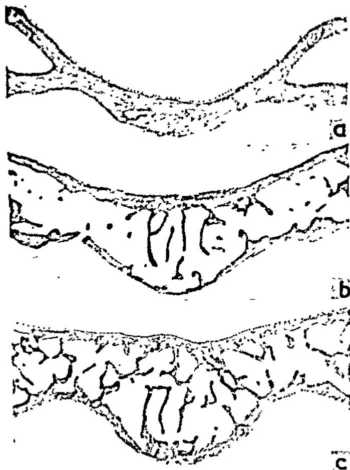


Fig 35 rDawings of coronal sections through basi-occipital bones of three litter-mate dogs. Duration of experiment 26–27 weeks Final age 33–34 weeks.

Black dots represent active osteoclasts, lines active osteoblasts.

(a) +A diet. Inner and outer bone tables have fused, with elimination of surfaces 2 and 3 (see Fig. 34) Surface 1, abundant active osteoclasts, especially towards middle line. Surface 4, a few scattered osteoclasts.

(b) −A diet. Bone still cancellous, with large marrow cavity Surface 1, some osteoblasts (reversal from same surface in (a). Surface 2, no cell activity Surface 3, no cell activity. Surface 4, a few active osteoblasts but otherwise no activity.

(c) −A diet for 24 weeks followed by +A diet for last 3 weeks of experiment. Surface 1, osteoclastic activity like (a), but much greater Surface 2, much osteoblastic activity. Surface 3, some osteoblastic activity Surface 4, a little osteoclastic activity, especially at periphery

Note. Reversal of action in (c) brought about by added vitamin A, especially on surfaces 1 and 2

in photomicrographs of mesial sagittal sections of the basi-occipital bone (Plate XVI). Vitamin A deficiency caused but little change on surfaces 3 and 4, so that the outer table of bone continued to grow normally and therefore to withdraw from the inner table, with the consequent enlargement of the marrow cavity and thickening of the bone.

The above facts apply to dogs killed when 20–30 weeks old, which, judged by their behaviour during life, had been depleted of vitamin A stores for 8 or more weeks. In older animals the rate of growth of the basi-occipital bone diminishes and the differences between the number and activity of osteoclasts and osteoblasts on surfaces 1 and 2 in +A and −A dogs, although still evident, were not as obvious as in the younger animals (Plate XVIII, a and b).

Drawings of coronal sections of the central portion of the basi-occipital bone of the older +A and −A littermates, which were 33 weeks old at death, can be seen in Fig. 35. In the +A dog (Fig. 35a), which had 5000 i.u. vitamin A daily, the inner and outer tables of bone have fused to form a compact structure and the marrow cavity has been eliminated. In the −A dog (Fig. 35b) the bone is cancellous, with a large marrow space, and surface 1 is almost flat as compared with that of the +A animal, which is modelled to the natural shape of the brain stem. It will be seen that on this surface in the +A dog there are still abundant osteoclasts which are more numerous in the centre than at the sides, a fact which may account for the hollowing near the mid-line. In the −A dog, on the other hand, there are osteoblasts at work, but no active osteoclasts. Thus, so far as surface 1 is concerned, the absence of vitamin A, as in the younger animal previously described, has reversed the type of bone cell at work. Surface 2, however, of the older −A dog (Plate XVIII) is different from that of the younger animal (Plate XVI) in that there

are no active osteoclasts. It is indeed interesting that the fusion of the inner and outer tables of bone in the +A dog appears to be accompanied in the corresponding −A animal by the disappearance of all active bone cells, both osteoclasts and osteoblasts, from surfaces 2 and 3 (Fig. 35b). In the case of surface 4, there are only a few scattered active osteoclasts left in the +A animal (Fig. 35a), whereas in the −A there are no osteoclasts and only a few active osteoblasts laterally (Fig. 35b) (Mellanby, 1947).

The basi-occipital bone of a litter-mate which was killed at about the same age, having received an A-deficient diet for 24 weeks of the experiment and, during the last 23 days of its life, vitamin A (5000 i.u. daily), is shown in the photomicrograph, Plate XVIIIc, and the drawing, Fig. 35c. Though this dog is referred to as a recovery animal, it will be seen that the shape (Fig. 35c) and texture of the bone have not become normal in the three weeks of vitamin A therapy; surface 1 is still flat and the bone cancellous. On the other hand, examination of the type and number of active bone cells on the different surfaces shows the great effort that is being made to bring about restoration of the bone to the normal shape. On surface 1 numerous active osteoclasts are apparently removing bone, while it is being laid down by the very active osteoblasts on surface 2, so that the inner table of bone is retreating to allow greater space for the brain stem (Fig. 35c). Surfaces 3 and 4 are

PLATE XVIII

Photomicrographs of coronal sections (× 90) of the inner table of basi-occipital bones of three litter mate dogs. Duration of experiment 26–27 weeks. Final age 33–34 weeks.

(a) +A diet Surface 1, some slight osteoclastic activity. Surface 2, inactive osteoblasts. (See Fig. 34 for plan)

(b) −A diet Surface 1, some slight osteoblastic activity. Surface 2, inactive osteoblasts.

(c) −A diet for 24 weeks, and then +A diet for last 3 weeks of experiment Surface 1, great osteoclastic activity. Surface 2, very strong osteoblastic activity. The osteoclasts and osteoblasts have again become very active in their normal positions.

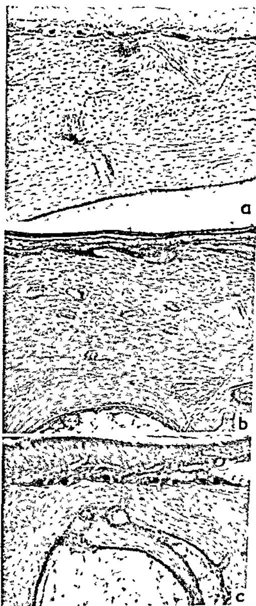


PLATE XVIII

also affected. Surface 3 shows osteoblastic activity, and on surface 4 active osteoclasts are seen, although there are many fewer than on surface 1.

It will be asked how soon after the addition of vitamin A to the diet the osteoclastic and osteoblastic activity returns to that seen in the young +A animal and how long such activity lasts. Insufficient evidence is as yet available to answer either of these queries fully, but it is known that 3 days after the addition of vitamin A to the diet cellular changes are negligible, but that after 13 days the osteoclasts and osteoblasts are rapidly correcting the abnormal growth of the period of vitamin A deficiency. This can be seen by comparing Plate XIXa, b and c. The three animals concerned were litter-mates. The +A and −A dogs were killed when 20 weeks old and from this time for 13 days the recovery animal was given 2000 i.u. vitamin A acetate daily. Plate XIXa is a photomicrograph of the basi-occipital bone (inner table) in the +A, Plate XIXb in the −A, and Plate XIXc in the recovery animal. From the intense reaction seen in Plate XIXc, it is obvious that this return of the bone cell activity towards the normal follows very quickly on the readministration of vitamin A. As has been seen above (p. 160), the evidence indicates that the basi-occipital bone ultimately becomes of normal shape.

PLATE XIX

Photomicrographs (× 95) of sagittal sections of inner tables of basi-occipital bones of three litter mate dogs. Duration of experiment 14–16 weeks. Final age 20-22 weeks.

(a) +A diet. Surface 1, few osteoclasts. Surface 2, many active osteoblasts (See Fig. 34 for plan)

(b) −A diet. Surface 1, many active osteoblasts. Surface 2, some osteoclasts.

(c) −A diet for 14 weeks and then +A diet for last 2 weeks of experiment Surface 1, many active osteoclasts. Surface 2, many active osteoblasts

Note: Removal of vitamin A from the diet has been followed by a reversal of cell activity at surfaces 1 and 2 (compare (b) with (a)). On adding vitamin A to the diet of a deficient animal the cells have again reverted to their normal position (c), but are much more numerous and very active.



PLATE XIX

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PLATE XIX

Photomicrographs (× 95) of sagittal sections of inner tables of basi-occipital bones of three litter-mate dogs. Duration of experiment 14–16 weeks. Final age 20-22 weeks.

(a) +A diet Surface 1, few osteoclasts. Surface 2, many active osteoblasts (See Fig 34 for plan)

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(c) −A diet for 14 weeks and then +A diet for last 2 weeks of experiment Surface 1, many active osteoclasts. Surface 2, many active osteoblasts.

Note: Removal of vitamin A from the diet has been followed by a reversal of cell activity at surfaces 1 and 2 (compare (b) with (a)). On adding vitamin A to the diet of a deficient animal the cells have again reverted to their normal position (c), but are much more numerous and very active.

(b) Sphenoid bone and the IInd nerve foramen

The general structure of the sphenoid bone in the region of the IInd nerve foramen is similar to that of the basi-occipital bone. There is an inner bony plate (surfaces 1 and

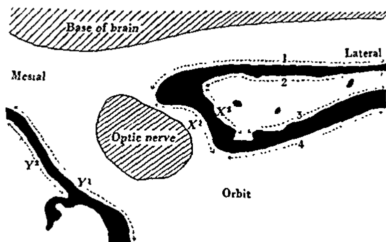


FIG. 36. Diagram of the general structure of the sphenoid bone at the level of the IInd nerve foramen. Surfaces have been numbered to simplify description in text.
Surface 1 is the bone surface adjacent to the brain.
Surface 2 is the marrow surface of the same inner table of bone.
Surface 3 is the marrow surface of the outer table of bone.
Surface 4 is the outer surface of the outer table of bone.
Surface X^1 is the lateral wall of the IInd nerve foramen and joins surfaces 1 and 4; similarly,
Surface X^2 is the marrow surface joining surfaces 2 and 3.
Surface Y^1 is the mesial wall of the IInd nerve foramen, and,
Surface Y^2 is the marrow surface of the same plate of bone.

2) adjacent to the brain and an outer bony plate (surfaces 3 and 4) remote from the brain, but in this case forming part of the orbit of the eye. Fig. 36 shows this formation. The sections to be described are coronal through the centre of the optic foramen, at right angles to the long axis of the head. They therefore show the mesial and lateral bone of the foramen wall, but not the anterior or posterior parts.

For ease in description, the bone of the lateral wall of the optic foramen has been divided into surfaces X^1 (adjacent to the second nerve and connecting surfaces 1 and 4) and X^2 (the marrow surface connecting surfaces 2 and 3). The mesial wall has been similarly divided into surfaces Y^1 and Y^2. Fig. 37a is a drawing of the optic foramen and sphenoid bone in a +A dog aged 33 weeks (26 weeks on diet). It will be seen that there is no osteoclastic activity on surfaces 2, 3, Y^1 and X^2, a little on surfaces 1, 4 and Y^2, with rather more on surface X^1. Active osteoblasts are confined to surfaces Y^1 and X^2. This would suggest that there is very little growth and moulding occurring at this age, with the possible exception of a little on the walls of the foramen. From the positions of the osteoblasts on surfaces Y^1 and X^2 and the osteoclasts on Y^2 and X^1, it is reasonable to suppose that there is still a small lateral movement of the foramen taking place at this age.

Fig. 37b is a drawing of a litter-mate of the same age, which had received an A-deficient diet for 26 weeks. It will be seen that osteoclastic activity has ceased on surfaces 1 and 4 and that osteoblastic activity (deposition) has taken its place on surface 4. Osteoclastic activity is seen on surfaces 2 and 3, so enlarging the marrow space. The absence of vitamin A has therefore brought about a thickening of the bone by reversing the osteoclastic and osteoblastic activity, as in the case of the basi-occipital bone. The actual mechanism of bone thickening in the present case is different from that described above in the basi-occipital bone. In the latter case it was surfaces 1 and 2, forming the bony plate adjacent to the brain, which were principally affected by the absence of vitamin A. In the thickening of the bone surrounding the optic foramen it is the bony plate remote from the brain (surfaces 3 and 4) which is specially affected. This thickening of the bone has lengthened the optic foramen. What, meanwhile, has been

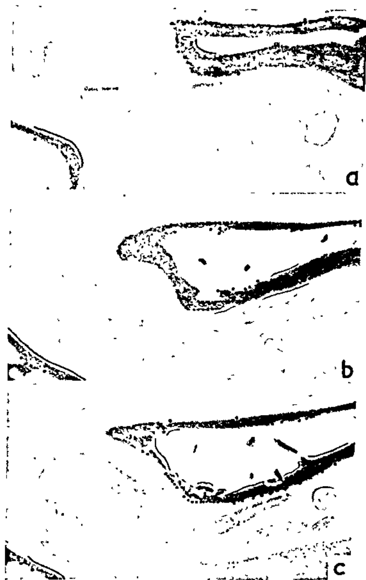


FIG 37

happening to its wall? Both growth processes, absorption and deposition, seem to have ceased on surfaces X^1 and X^2 in the −A animal, while growth processes have continued in a normal way on the lengthened surfaces Y^1 and Y^2. This would have the effect of narrowing the foramen, for, while the mesial surface would move laterally, the lateral surfaces would remain stationary, thereby constricting the nerve and vessels passing through the foramen.

If we now examine the recovery animal (Fig. 37c), a third litter-mate which was maintained on an A-deficient diet from the age of 7 to 31 weeks and then received 5000 i.u. of vitamin A daily for 3 weeks (23 days), we see that there is a return to the normal as regards position of active cells. Surfaces Y^1 and Y^2 again show no variation in type of cell, although there may be some increase in intensity of growth, Surfaces 1 and 2, which showed comparatively little change due to A deficiency, also show but little change on the return of the vitamin. The greatest response to the addition of the vitamin is seen on surfaces 3, 4, X^1 and X^2. There is obviously a great eating away of bone from surfaces 4 and X^1, with a correspondingly great deposition

Fig. 37 Drawings of coronal sections through the sphenoid bones of three litter-mate dogs. Duration of experiment 26–27 weeks Final age 33–34 weeks.

Black dots represent active osteoclasts; lines active osteoblasts.

(a) +A diet. Very little cell activity on surfaces 1, 2, 3 and 4. (See Fig. 36.) Osteoclasts are seen on surfaces Y^2 and X^1, osteoblasts on surfaces Y^1 and X^2.

(b) −A diet. No changes in cell activity on surfaces Y^1 and Y^2. Cessation of activity on X^1 and X^2. Surface 3 shows both osteoclastic and osteoblastic activity and surfaces 2 and 4 show reversal of cell activity.

(c) −A diet for 24 weeks, followed by +A diet for last 3 weeks of experiment. No changes on surfaces Y^1 and Y^2. Activity on surfaces X^1 and X^2 has returned to the normal type, with an increase in intensity. Surfaces 3 and 4 show a great activity of bone cells and a reversal to the normal type. Surfaces 1 and 2 show little change.

on surfaces 3 and X^2. It seems obvious that continuation of these processes will restore the foramen to the normal (+A) diameter, reduce its length by reducing the thickness of the sphenoid bone, and correct its position by moving it laterally.

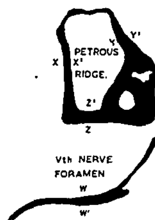


FIG. 38. Diagram of petrous ridge and Vth nerve foramen. Surfaces have been numbered to simplify description in text.
Surface X is the mesial surface of the petrous ridge.
Surface Y^1 is the lateral surface.
Surfaces Z and W are the boundaries of the Vth nerve foramen.
Surfaces X^1, Y, Z^1, and W^1 are the corresponding marrow surfaces.

(c) *Petrous ridge and the Vth nerve foramen*

To simplify the description here, reference may be made to Fig. 38, which is a diagrammatic representation of the region under discussion. Examination of serial sections of the petrous ridge and Vth nerve foramen of normal animals has shown that there is absorption of bone from surfaces X and Y by osteoclasts and a corresponding deposition on surfaces X^1 and Y^1 by osteoblasts, suggesting that during normal growth there is a moving apart of the petrous ridges. This may also, however, be affected by a change in size of the basi-occipital bone, which acts as a spacing-piece

between the ridges. Examination of surfaces Z, Z[1], W and W[1] indicates that absorption normally predominates on Z and W and deposition on Z[1] and W[1]. The effect of these activities of bone cells would be to enlarge the Vth nerve foramen and to allow comfortable room for the growing nerve.

It has been shown in the case of other bones that the number and the intensity of activity of the bone cells are normally reduced as the animal grows older and the same applies to bone in the region of the petrous ridge. Fig. 39a is a drawing of a section from a +A animal aged 33 weeks, showing the active bone cells. It is apparent that even at this age there is still some osteoclastic action within the Vth nerve foramen, tending to enlarge it. Having seen the method of normal growth here, it is now possible to follow the changes caused by vitamin A deficiency. This is shown in Fig. 39b, which represents the appearance of a litter-mate of the same age which had received an A-deficient diet for the last 26 weeks of its life. Osteoclastic action has almost disappeared and been replaced by osteoblastic activity, with obvious ill effects on the Vth nerve. A third litter-mate of approximately the same age, which had received the vitamin A-deficient diet for 24 weeks and then during the last 3 weeks of its life had had a daily ration of 5000 i.u. of vitamin A, is shown in Fig. 39c. The reaction is clearly great. Surfaces Z and W, which in the normal showed a little osteoclastic activity and in the A-deficient animal some osteoblastic activity, here show a tremendous osteoclastic activity. Surfaces X and Y, which in the +A animal showed no activity and in the −A animal osteoblastic activity, now show great osteoclastic activity. Thus it is obvious that in this region, as in the others described, vitamin A deficiency causes a change of type of bone cell activity, which returns to the normal type when vitamin A is added to the deficient animal's diet.

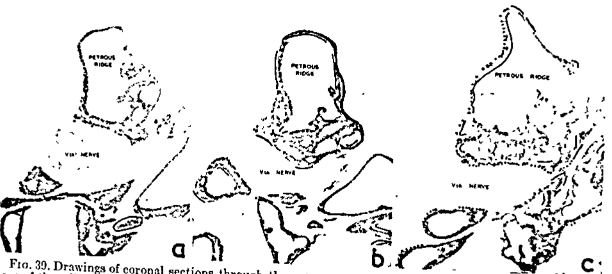


Fig. 39. Drawings of coronal sections through the petrous ridge and Vth nerve foramen of the temporal bone of three litter-mate dogs. Duration of experiment 26–27 weeks. Final age 33–34 weeks.

(a) +A diet. Except for a few osteoclasts on surfaces Z and W (see Fig. 38 for plan), there is very little activity in this section.

(b) −A diet. Osteoblastic activity is now predominant even on surfaces Z and W, although two osteoclasts are still seen on surface Z.

(c) −A diet for 24 weeks followed by +A diet for last three weeks of experiment. Great osteoclastic activity on surfaces X, Z and W, rather less on surface Y. Strong osteoblastic activity on surfaces X^1 and Y^1 to balance the removal of bone from X and Y.

(d) *Labyrinthine capsule and the internal auditory meatus*

The bony labyrinth is probably fully formed at the time the special feeding in these experiments begins (6–7 weeks). At this age also the periosteal bone covering the labyrinth on the side adjacent to the brain is nearly fully formed, as is indicated by the few and inactive bone cells on its surface. When the periosteal surface of the labyrinthine capsule is compared with surface 1 of the basi-occipital bone in animals about 20 weeks old, it is seen that, whereas there is much osteoclastic activity on the basi-occipital bone, only a few inactive osteoclasts and osteoblasts are found on the labyrinthine bone, indicating that at this age bone growth has almost ceased in this position.

In an A-deficient animal of this age these osteoblasts become very active, so that, whereas in the case of the basi-occipital bone the change produced by A-deficiency is mainly, although not entirely, due to a failure to absorb bone, the outstanding character of the same deficiency on the labyrinthine capsule is to produce excessive laying down of bone. As previously shown (Mellanby, 1938), parts of this capsule may in A-deficient animals become so thickened that they largely block the internal auditory meatus, which may be elongated and narrowed (Plate Vb). In the younger animals the extra bone formed is cancellous, with large fatty marrow spaces. Fewer spaces are seen, however, as the animals grow older, until at 33 weeks of age (Fig. 40b), after 26 or so weeks of the A-deficient diet, the bone is relatively compact. The marrow spaces of the younger, but rarely of the older, animals sometimes contain a few active osteoclasts.

In the −A animals the thickened periosteal bone adjacent to the brain increases the distance from the helix to the medulla, lengthens the internal auditory meatus and

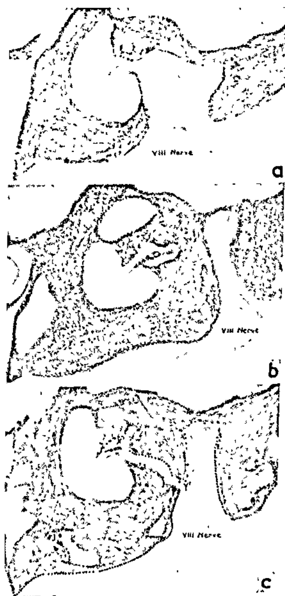


FIG. 40.

causes stretching of the VIIIth nerve. Within the internal auditory meatus there is a change in the position of bone cells reminiscent of that of the basi-occipital bone, namely, slight osteoclastic activity on the surface adjacent to the VIIIth nerve in the +A dog becoming in the −A animal osteoblastic activity, with active osteoclasts on the marrow surface (equivalent to surface 2 of the basi-occipital bone). There is a definite deposition of bone within the meatus, which causes compression and, as it is not laid down equally, but is found rather in nodules, tortuosity of the nerve.

Fig. 40a and b are drawings of the labyrinthine capsules of the +A and −A litter-mates which were 33 weeks old when killed. The increased thickness of the bone in the −A dog (Fig. 40b) is clear. There are practically no active osteoblasts or osteoclasts in either series of sections, thus indicating that both normal (Fig. 40a) and abnormal growth (Fig. 40b) have ceased. In Fig. 40a the few active osteoclasts seen are on the surface of the internal auditory meatus, which suggests that it is still necessary at this age to have a mechanism at work capable of modifying its size and shape.

If the absence of active osteoblasts and osteoclasts from the labyrinthine periosteal bone in the +A and −A

FIG. 40. Drawings of coronal sections of labyrinths of three litter mate dogs. Duration of experiment 26–27 weeks. Final age 33–34 weeks.

Black dots represent active osteoclasts, lines active osteoblasts

(a) +A diet. Practically no active bone cells visible except a few osteoclasts on the surface of the internal auditory meatus.

(b) −A diet. No active cells visible. In the internal auditory meatus there has been a great formation of new periosteal bone, which has squeezed the VIIIth nerve: the increased activity of osteoblasts which produced the overgrowth has ceased.

(c) −A diet for 24 weeks, followed by +A diet for last 3 weeks of experiment. The restoration of vitamin A to the diet has brought about a tremendous reaction and active osteoclasts in large numbers are now seen, apparently removing the superfluous bone.

Note: The layer of osteoclasts on the surface X^1 Y^1 (see Fig. 41) in c

animals of this experiment suggests absence of control by vitamin A, it is only an illusion. Proof of this can be seen in the labyrinthine capsule of the third litter-mate of the same age (Fig. 40c), which was on a diet deficient in vitamin A for 24 weeks and then received for the last 3 weeks (23 days) of its life 5000 i.u. of vitamin A daily. The reaction on the addition of vitamin A is clearly tremendous, the surface of the periosteal bone being covered with active osteoclasts (Fig. 40c) which were obviously there in an attempt to restore the bone to its normal (+A) shape (Fig. 40a).

There appears to be a definite plan in this absorption of excess bone, since great osteoclastic activity is found wherever osteoblasts have been active during the period of A deficiency. Thus the whole surface of the periosteal bone adjacent to the brain and within the internal auditory meatus is covered with active osteoclasts in the recovery animal (Fig. 40c). Since all this periosteal bone overgrowth is due to the excessive activity of osteoblasts during the −A period, this result is in keeping with the general recovery reaction. One point of special interest is seen in the recovery animal, namely, the presence of abundant osteoclasts on a surface marked X^1Y^1 in the sketch (Fig. 41), and the almost complete absence of these cells from the opposing surface XY. Although the portion of bone marked M is of periosteal origin, like that marked P, it seems probable that the mode of growth of M is rather different from that of P. A possible difference is suggested in the sketch, and it will be seen that M may grow in two directions, ZY^1 and X^1Y^1, whereas P probably grows in one direction only as bony layers deposited on surface XX^2. This explanation of the method of bone growth during the −A period (Fig. 40b) would allow the appearance of osteoblasts on surface X^1Y^1 of the recovery animal (Fig.

40c) to be regarded as in line with the usual recovery reaction in regard to osteoclasts.

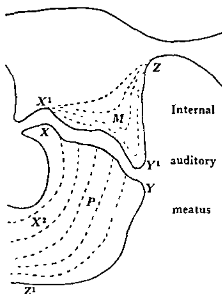


Fig. 41. Sketch of internal auditory meatus and overgrown periosteal bone due to vitamin A deficiency, to illustrate the way in which bone is apparently laid down in M and P. (c. f Fig 40.) It will be seen that in M the bone is added on both surfaces ZY¹ and Y¹ X¹.

Bone P, however, is laid down in the lines indicated, and the surface of growing bone faces in one direction only. It is usual in recovery animals to see osteoclastic action where, in the −A, osteoblastic action has been predominant. Thus surfaces Z Y¹, Y¹ X¹ and Y Z¹ would be expected to show much osteoclastic activity and surface Y X to show little or none.

It seems legitimate to assume from this experiment that the apparent absence of active osteoclasts and osteoblasts on the normal labyrinthine capsule at this age does not mean that vitamin A has ceased to control, directly or indirectly, either the bones or the bone cells, but that it

animals of this experiment suggests absence of control by vitamin A, it is only an illusion. Proof of this can be seen in the labyrinthine capsule of the third litter-mate of the same age (Fig. 40c), which was on a diet deficient in vitamin A for 24 weeks and then received for the last 3 weeks (23 days) of its life 5000 i.u. of vitamin A daily. The reaction on the addition of vitamin A is clearly tremendous, the surface of the periosteal bone being covered with active osteoclasts (Fig. 40c) which were obviously there in an attempt to restore the bone to its normal (+A) shape (Fig. 40a).

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how this pattern depended on the relative position and degree of activity of the bone cells, osteoblasts and osteoclasts. In keeping with this, although the effects of vitamin A deficiency and of its subsequent restoration to the deficient diet varied at each point in accordance with the primary pattern of growth, the outcome of vitamin A deficiency was usually to produce a more abundant cancellous bone which embarrassed the nerve in close proximity while that of a vitamin A restoration was a cell arrangement calculated to bring back the malformed bone to a normal shape. In spite of the foregoing analysis, to generalize as to the effect of vitamin A on osteoblastic and osteoclastic activity on all growing bone is still difficult, but this point is considered at greater length in Chapter X. Nor, as stated earlier in the present chapter, is it certain what is the ultimate effect on the shape of the malformed bones of the restoration of vitamin A. The early cell changes always seem to indicate the rapid recovery of bone shape and this in many cases certainly does happen. There are, however, other instances, such as the supra-occipital bone mentioned above, where it looks as if the bone may remain abnormally shaped.

4. Summary

(1) The addition of vitamin A to the diets of A-deficient animals during growth initiates recovery processes with the result that:

(a) The ataxia and incoördination of movement is considerably reduced and may almost disappear in early cases, but in more pronounced cases complete recovery does not take place.

(b) The pressure effects on the cerebellum and brain stem are relieved, and the shape of these parts of the central nervous system becomes more normal.

(c) The papilloedema, due to the increased intracranial

holds a guiding hand on these cells so that they produce and mould periosteal bone covering the labyrinth only in amounts and shape compatible with the easy passage of the VIIIth nerve from the labyrinth to the central nervous system. If, during the absence of the vitamin, the periosteal bone grows so that excessive tissue is laid down, then the return of the vitamin to the body not only reverses the process but causes an exaggerated response of bone cells calculated to clear away the excessive bone formation and bring its shape quickly back as near to the normal as possible.

Comparing the reactions of the labyrinthine capsule and the basi-occipital bone in A deficiency, it is seen that the first is an example of excessive bone formation following the loss of vitamin A, the return of the vitamin causing removal of the hyperplastic new bone. In the second, loss of vitamin A is followed primarily by cessation of removal of bone already laid down, although in the earlier weeks of the experiment there may be in addition a small deposition of new bone; on the return of the vitamin the bone removed by the hosts of active osteoclasts is for the most part bone which, under normal conditions, would have been removed earlier.

Detailed accounts have now been given of the position and intensity of action of osteoblasts and osteoclasts during normal growth at: (a) the basi-occipital bone, (b) the sphenoid bone and the IInd nerve foramen, (c) the petrous ridge and the Vth nerve, and (d) the labyrinthine capsule and the internal auditory meatus. The changes in position in number and in intensity of activity of the bone cells at each of these positions when vitamin A was removed from the diet were described, and finally how these same cells reacted when vitamin A was restored to the defective diet. These different places were examined in order to show how varied was the normal pattern of bone growth and

optic foramen. The recovery changes on adding vitamin A are also different in each case.

(6) The altered shape of bones adjacent to the nervous system may destroy cranial and spinal nerves and exert other harmful effects, but the recovery changes in dysplastic bone on the addition of vitamin A to the diet take place independently of the condition of the adjacent nerve or nervous tissue.

(7) Long bones, bones of the face and pelvic bone—indeed probably all the bones of the body—are similarly affected by vitamin A deficiency, so that they become coarser and thicker and are no longer delicately moulded. The practical consequences of malformation of these bones are probably not very important from a functional angle but their strength may be decreased, and their shape may not be aesthetically satisfactory.

pressure, recedes. The optic disc may ultimately appear normal.

(d) Deafness is probably the commonest remaining defect in recovery animals.

(2) The foregoing more detailed experimental evidence supports the view expressed in Chapter VIII that the general function of vitamin A as regards growing bone is to control its shape, and especially its fine moulding, by influencing the position and the activity of osteoclasts and osteoblasts.

(3) Recovery changes following the addition of vitamin A to the diet of an A-deficient animal confirm this view, since the vitamin brings about a return of osteoclastic and osteoblastic activity to the surfaces where it is normally found. This reaction is often of a very intense nature and its object is clearly an attempt to restore rapidly the normal shape of the malformed bone produced during the deficient period. The subsequent history of these bones is not yet certain, but some return to their normal shape while others probably do not.

(4) The change in position of activity and in number of osteoclasts and osteoblasts resulting from vitamin A deficiency is orderly, not anarchic, and is usually a modification or even a reversal at the effective surfaces, deposition of bone taking place where there was previously either absorption or no bone cell activity, whereas, at other surfaces, bone absorption is substituted for deposition of bone or no bone cell activity.

(5) Although vitamin A deficiency causes a general thickening and dysplasia of bone by its effect on osteoclasts and osteoblasts, the method of producing this abnormal state varies, as is shown in the three regions discussed, namely, the basi-occipital bone, the periosteal bone covering the labyrinth, and the sphenoid bone in relation to the

able as working hypotheses. It is even worse when the speculation becomes a working hypothesis only to be rejected after months or years of further work.

One other general remark may be permitted. It is obvious that there is no end to an investigation of this nature. The final results described above will no doubt form the basis for other work. The facts as described will probably be for the most part, correct, but it is rare for biological discoveries to be interpreted in the right light or in their true perspective in the early stages. There is no reason to believe that the present work will differ in this respect from many other advances in biological knowledge.

The foregoing description of the actions of vitamin A, and the effects of its absence from the body and of its return to the diet of an A-deficient animal, shows clearly that this substance plays an important part in controlling bone growth, shape and texture. It is made clear that normally in the case of bones adjacent to the growing nervous system, even the finer degrees of moulding have to be specially adjusted. Thus, the precise control of bone development by a humoral agent is of the utmost importance to the animal's life and well-being. As the nervous system and its branches grow, the cranial and spinal cavities and the foramina which allow the passage of nerves to and from the central nervous system must enlarge to accommodate these tissues. If vitamin A is deficient in the growing puppy, bone development is so changed that the skull cavity and the spinal canal enlarge inadequately and irregularly, and some of the foramina even become smaller, with disastrous results, including degeneration of the olfactory, optic, trigeminal and auditory nerves, the posterior roots of the spinal cord and many neurones in the brain stem and spinal cord.

If, after a period of some weeks, the vitamin is restored to the diet, there is a vigorous attempt to correct the wrong

Chapter X

DISCUSSION ON VITAMIN A DEFICIENCY AND INCOÖRDINATION OF MOVEMENT

An account has now been given of the investigations which were made to solve the problem set out in chapter I, namely to find the cause of the ataxia and incoördination of movement that developed in growing animals fed on diets which, so far as was known, were complete except for vitamin A and carotene. An attempt has been made to describe the development of the research and the gradual unravelling of the factors involved as the work progressed. While it is probably easy to judge from the preceding chapters where and when progress was made and generally to group and evaluate the positive results, it cannot be easy to understand the doubts, difficulties, disappointments and mistakes that have been major factors in work which has continued intermittently over a period of about 25 years.

Chapter III was devoted to a brief account of experiments which failed to open up the problem, but the narrative would hardly bear a full review of all the other will-o'-the-wisps that were pursued in the course of the work. Yet, failure fills a much larger part in biological investigation of this nature than in the more scientifically developed chemical and physical fields. The solution of a biological problem when worked out often seems so reasonable and so simple that wonder is expressed that any other sequence of events could be contemplated, but those who think along these lines can have had little experience in opening up new fields of biological enquiry. Such work involves constant speculation and formulation of ideas, most of which, on testing, do not reach the stage of being accept-

A deficiency may have some significance, but it is unlikely to prove as important in parturition as that produced by vitamin D deficiency. In the case of the facial bones mention has been made (p. 126) of the enlarged malar bone which encroaches on the lower margin of the orbit to a greater degree than in the normal animal. This may cause some embarrassment of the eyeball but it will not produce changes in vision comparable to those following pressure on the optic nerve as it passes through the sphenoid bone. The change in shape of the malar bone may also alter the facial expression.

Although the general result of these changes on bone growth and shape is obvious, the problem becomes more difficult when an attempt is made to generalize in terms of the reactions of osteoclasts and osteoblasts to vitamin A. One of the difficulties is the variation in intensity and manner of bone growth at different ages and in different bones at a given age. In, for instance, the basi-occipital bone of a +A puppy aged 20 weeks, bone cell activity is greater than in an animal on the same diet aged 30 weeks. Correspondingly the abnormal cell activity in −A animals is greater at 20 than at 30 weeks of age. If, however, vitamin A is added to the diet of a deficient animal when 30 weeks old, the bone cell activity returns to the normal type, but the intensity is greater than that seen even in the normal 20-weeks +A animal, and therefore much greater than in the normal +A animal of 30 weeks. Then there is the further difficulty that the mode of bone growth in different parts of the skeleton varies. For instance, in the basi-occipital there is normally osteoclastic absorption on surface 1 (Fig. 34) and osteoblastic deposition on the marrow surface (surface 2) whilst on the other hand, there is deposition on the outer surface and absorption on the marrow surface of the diaphysis of the femur. Differences such as these and others of a lesser degree frequently occur, especially in

growth of the deficient period. This fact strengthens the evidence that vitamin A exerts a powerful controlling influence on the elements responsible for coördinated bone growth and that it thereby is a factor in bringing about the necessary adjustment between the shape of growing bone and that of the developing nervous tissue. Whether its effect is direct or indirect, and to what extent the control continues beyond the period of active growth in early life is not certain. It is known, however, that the addition of vitamin A to the diet of a −A animal at a time when there is no bone cell activity (Fig. 40b) produces on the surface of the labyrinthine capsule a vigorous osteoclastic activity (Fig. 40c). Evidence has also been given to show that in adult dogs the removal of vitamin A from the body causes a very slow overgrowth in that part of the periosteal bone of the labyrinth forming the internal auditory meatus (Plate XIa), in which bone cell activity, and it would therefore be expected growth, has ceased. These results suggest that the control of bone cell activity by vitamin A probably does extend beyond the period of active growth.

Reference has already been made (see p. 125) to the fact that it is not only bones near the nervous system that are moulded by the action of vitamin A but that others remote from these tissues, such as those of the face and pelvis and the long bones, are also so controlled, for in the absence of vitamin A they lose their normal finely moulded shape and become coarse and blunted. What such a change in shape means in terms of the physiology of the long bones and their adjoining tissues cannot be stated, but it is certainly not so serious as the destruction of, or other pathological changes in, the nervous tissue following the dysplasia of skull and vertebral bones. The long bones are probably weakened and the loss of shapely appearance of wrists and ankles may be of aesthetic importance. The pelvis of reduced internal dimensions produced by vitamin

The following summary of changes due to the presence and absence of vitamin A applies to the special examples mentioned above and possibly to all growing bones.

(1) Neither in the presence nor in the absence of vitamin A are active osteoblasts and active osteoclasts intimately mixed at any one part of a surface of a table of bone, although the activity of either may be much greater at one part of a surface than at another. For instance, the place where a muscle is inserted into a bone surface is more likely to be the site of a greater number of osteoclasts than another point of the same surface where no muscle is inserted. There are rare occasions also when one portion of a surface shows the laying down of bone and another part of the same surface shows bone removal, but when this happens the parts affected are distinct (See Fig. 37b).

(2) When osteoclastic activity is evident on one surface, the opposing surface of the same table of bone is usually covered with active osteoblasts. This type of coördination is clearly necessary, since otherwise either the bone would disappear and the marrow would be exposed or the marrow cavity would become full of bone and there would be no growth or movement. In recovery cases, local exposure of bone marrow does happen occasionally over very small areas, but it is probable that these areas of bone weakness are soon strengthened. This coördination of osteoclasts and osteoblasts is one of the ways by which the basi-occipital bone is moved away from the base of the brain, thereby allowing expansion of the posterior fossa, and is also the method by which the optic foramina are moved away from the middle line during normal growth or during recovery from vitamin A deficiency.

(3) The effect of removing vitamin A from the animal body is usually to stop osteoclastic activity on the most effective surfaces, for example, in the basi-occipital bone

bones of complicated structure and only when the objective of bone growth and the means of procuring this at any particular site are understood, can an appreciation of the effect of vitamin A on the local growth be obtained. By examining the abnormal bone growth, and perhaps more easily by noting the efforts to correct it that follow the addition of vitamin A to the diet of a deficient animal, the mechanism of normal growth and the method by which vitamin A controls bone growth at any point becomes clearer.

It might be asked why it should be assumed that the reactions of bones to vitamin A deficiency and to its later addition to the diet indicate the normal way in which a particular bone grows. The answer is that in the case of bones adjacent to nervous tissue and to the central nervous system in particular, the absence of vitamin A in most cases results in bones of abnormal shape which either destroy or threaten to destroy the nervous tissue. The bone change which follows the restoration of vitamin A to the body is in the direction of a return to normal shape or at least to a shape which brings relief of pressure to the nervous tissue. In such cases, therefore, it seems legitimate to assume that the recovery reaction of bone indicates in an exaggerated form the normal growth process.

So far as is known, the only cells responsible for bone growth are osteoblasts and osteoclasts, but it has been seen above that Nature uses these two types of cells as regards position, number and degree of activity in many combinations at different points of growth. In three of the examples given in chapter IX, e.g. the basi-occipital, the sphenoid bone around the optic foramen, and the labyrinthine capsule, it was evident that the normal manner of growth varied and in the same way, although vitamin A deficiency and recovery therefrom had the same general results, disposition of the bone cells was different in each case.

in accordance with the size and growth of the citadel to be protected. The central nervous system is a citadel and as it grows the protecting walls are normally moved farther out. As the bones of the skull and vertebrae grow bigger the space they surround is enlarged by absorption of their inner surface and deposition on their outer surface. Now if the director (vitamin A) of building operations disappears, it might then be expected that the brick layers (osteoblasts) and demolition squad (osteoclasts) would either go on strike or work in a completely disorderly way, but this does not happen in the bony ramparts. It is rather as if the place of vitamin A as a wise director of operations were taken over by that worst kind of director—the energetic man with no wisdom, whom we all know so well nowadays—who says: "I am going to show you how things should be done; now you will see something really happen." His directions are: "You must work harder than ever, but in a different way. You builders (osteoblasts) must lay down bricks wherever there is a foundation (periosteum). You demolishers (osteoclasts), working nearest the citadel, must leave that position and continue your labours elsewhere."

The result is that the walls, instead of enclosing a greater area as the citadel grows, now encroach on the nerve control stations, lines of communication and the administrative centres and squeeze all the vital structures into so small a space that work inside the citadel is impossible. Parts of the citadel (the central nervous system) are destroyed and the city (animal) with it. There has been no slacking and no anarchy among the building operatives but, by working at the wrong place and in the wrong direction, they have converted a protective structure into one of destruction. Thus, vitamin A, by regulating the activities of the bone cells in this position coördinates a beautiful adjustment of bone and nervous system growth. How important this function is to animal development can

the surface near the nervous tissue. If the osteoclastic action has been great in the presence of vitamin A, then the removal of the latter usually results in cessation of or greatly reduced activity at that surface. If the osteoclastic action at the time the deficiency is established is slight, then the removal of vitamin A is more likely to result in osteoblastic activity, whilst a surface on which there is normally little or no bone cell activity would in an A deficient animal be expected to show a vigorous laying down of bone.

(4) Although the absence of vitamin A during bone growth may cause devastating effects on the nervous system, its removal from the diet is not synonymous either with cessation of bone cell activity or with anarchy of these cells. In the presence of vitamin A the natural processes of osteoclastic and osteoblastic activity at each place seem to be pre-determined and, the orders having been given, vitamin A sees that they are carried out. If the vitamin is absent, growth goes awry, since the orders are not *only* disobeyed but changed in a more or less systematic way.

Although it is clear that vitamin A has a most important task in ensuring the normal moulding and shaping of bones, it cannot have escaped attention that what the osteoblasts and osteoclasts do at any position of bone growth to bring about this shape is only partially determined by this vitamin. The factors that control the normal growth of bone—the normal pattern of growth—at each point are, it is true, affected by vitamin A, but they in turn— whatever they are—control the kind of effect vitamin A has, and what happens in its presence, its absence and its restoration after absence.

It will be generally agreed that Nature has done well to provide the central nervous system with a strong bony protection. Its safety from assault is essential both for the survival of the individual and the race. Those who build the walls and ramparts must, however, plan their activities

Appendix I

VITAMIN A INVESTIGATION

1. EXPERIMENTAL DIETS

The basal diets, which had to be such that they were compatible with good health and growth, were composed for the most part of ordinary foodstuffs. It was regarded as important that all the animals in a given experiment should eat the same amount of the basal diet. If any fell behind in its capacity to eat up its daily ration, either this animal was discarded or the whole experiment was terminated at that point.

(a) *Rabbits*

Litters of rabbits from 8 to 10 weeks old were given the following basal diet which was deficient in carotene and vitamin A:

Oats and bran (4:1), 40–70 g.,
$CaCO_3$, 1.5% of oats and bran mixture,
Dried, heated and oxygenated alfalfa, 10 g.,
Vitamin D_2 as irradiated ergosterol, 200 i.u.,
Vitamin C as decitrated lemon juice (in some experiments), 1 ml.

The alfalfa in the above diet was ground to a fine powder, heated for 36 hours at 120°C in thin layers on trays in an electric oven, and stirred frequently during the heating process in order to allow full exposure to the air. By this means its carotene content could be entirely destroyed. There must still, however, have been a trace of carotene in the oats and bran.

One or more rabbits of each litter were protected from the effects of vitamin A deficiency by the addition to their diet of unheated alfalfa, cabbage, carrot or some other source of carotene or vitamin A

(b) *Dogs*

Litters of puppies from 6 to 10 weeks old were fed on basal diets of the following type:

Cereal (flour, oatmeal, etc.) 50–300 g.,
Separated milk powder, 20–30 g.,
Lean meat, 15–20 g ,

be appreciated by the drastic and dramatic effects produced when the mechanism goes wrong in the absence of the vitamin.

In setting out the problem at the beginning of these lectures it was stated that the cause of the incoördination in young animals was independent of, but in some way related to, the cause of rickets. It was soon recognised that the incoördination was due to a deficiency of the vitamin A moiety of the original fat-soluble complex used in the rickets investigation, and that the condition was associated with widespread degeneration of the central and peripheral nervous systems. It was thought at that time that the nerve degeneration was probably a *direct* reaction to the absence of vitamin A from the animal body. Later, however, it became clear that this was not the case, but that most of the degeneration could be ascribed to the fact that *a function of the vitamin A moiety was to control* the activity of osteoblasts and osteoclasts so that in its absence from the body, the bones were thickened and altered in shape, *and that the incoördination was, in fact, due largely*, if not entirely, to the pressure on the nervous system of this thickened bone. Rickets, on the other hand, is due primarily to a deficiency of the vitamin D moiety, whose main function is to harden the bones by controlling the deposition of a calcium-phosphate compound in the osteoid tissue laid down by the osteoblasts. Thus both these vitamins, working in close association, play an important part in bone growth—the one influencing the shape, and especially the moulding of growing bone, the other the calcification and hardness.

It is now clear how different and yet how closely related are these two problems of rickets and incoördination. Further investigation will no doubt show that the solution is not so simple as suggested and that other chemical agents play important parts in the scheme.

Peanut oil, 5 ml.,
Baker's yeast, 0.5–2.5 g,
Vitamin D_2 as irradiated ergosterol, 10 i.u.

In each litter one or more animals were given in addition, by pipette, vitamin A (200 i.u.) daily.

(d) *Rats*

Litters of rats were given 2.5 g., increasing to 20 g. of the following diet when 3 to 4 weeks old:

Diet 43

Oatmeal, 89 g.,
Heated and oxygenated alfalfa, 2 g.,
NaCl, 0.85 g.,
$CaCO_3$, 1.5 g.,
Sodium pyrophosphate, 1.65 g.,
Food yeast, 2.37 g.

This diet, like that given to other animals, was not completely devoid of carotene, but it allowed depletion of body reserves. Vitamin A and vitamin D oils were either given by pipette or mixed with the food.

The synthetic diet used was as follows:

Casein, heated and oxygenated,	18 g.,	3–15 g. per day.
Cornstarch	60.9 g.,	
Osborne and Mendel salt mixture (1913)	3.0 g.,	
Food yeast (dry),	2.5 g.,	
or Baker's yeast	10.0 g.,	
Peanut Oil	8.0 ml.	
Vitamin D_2	10 i.u. per day.	

This diet is similar to, but not identical with, that used by Wolbach and Bessey (1941). The casein was heated and oxygenated in the same manner as the alfalfa described on p. 195.

(e) *Chicks*

From the time of hatching, chicks were given ad lib, the following vitamin A and carotene deficient diet:

Oatmeal	49.5 g.
Bran	20 0 g.
Alfalfa (heated and oxygenated)	10.0 g.
Food yeast	10.0 g.
Separated milk powder	10.0 g.
$CaCO_3$	0.5 g.

NaCl, 1-2 g.,
Baker's yeast, 2.5-15 g.,
Peanut or olive oil, 10 ml.
Vitamin D_2 as irradiated ergosterol, 1000-2000 i.u.,
Ascorbic acid, 5 mg.

(In some early experiments 5 ml. orange juice was given instead of ascorbic acid.)

Points worthy of note in connection with this diet are:

(1) The cereal, unless made into bread, was cooked in a pressure steamer (½ lb. pressure) for 1½ hours.

(2) Yeast was mixed with water and boiled before being added to the rest of the food.

(3) The meat was hand-cleaned by dissection to remove all visible fat. Usually raw horse flesh was given, but occasionally beef was used, and this was cooked. All the animals in one litter had meat of the same kind treated in the same way.

This diet was not completely devoid of either vitamin A or carotene. There was a trace of carotene in the cereal, while the meat probably contained a little vitamin A, but the amount was not sufficient to prevent depletion of the body. Under the conditions of these experiments the calcium in the diet was not optimal for bone formation in the early months when growth was rapid; increasing the calcium modified, but did not prevent, the effects described in −A animals. In each litter one or more animals were given supplementary vitamin A or carotene (1000–5000 i.u. daily) as vitamin A acetate, mammalian liver fat, cod-liver oil or steamed cabbage. The form in which the vitamin A or carotene was given did not appear to matter, and, so long as sufficient was absorbed from the alimentary canal, no ataxia was produced, normal bones resulted and the nervous system did not show characteristic degenerative changes.

To ensure that a dog received its prescribed amount of vitamin D and/or vitamin A, these, when given in the form of oil, were not mixed with the food but were given to the dog by pipette during the period of digestion.

(c) *Ferrets*

Litters of animals 5–8 weeks old were fed on diets similar in composition to those given to the dogs:

Cereal (oatmeal, bread), 10–50 g.,
Separated milk powder, 5 g.,
Lean meat, 7.5–10 g.,

with alcohol and glycerin, and then teased before microscopic examination. This method is only useful in the case of peripheral nerves, in which it shows up the changes very beautifully (Plate I, c and d).

In order to determine the position of complete disappearance of groups of nerve fibres, Kulchitzky's modification of the Weigert-Pal method was adopted.

(b) *Nerve cells*

The chief fixatives used for nerve cells were 10 per cent neutralized formol saline and Carnoy's solution (Perdrau's modification). Of these Carnoy's gave the better results owing to more rapid penetration due to the presence of acetic acid, the subsequent staining being deeper and sharper as well as more lasting.

The stains employed for demonstrating the changes seen in nerve cells included: methylene blue (polychrome), toluidene blue and the Unna-Pappenheim stain (pyronin and methyl green). Silver impregnation methods were little used in these experiments although there were indications that such methods would have given interesting results.

(c) *Bone and other tissues*

In many experiments, and especially where it was desired to examine the nerve together with the surrounding bone, the animals were injected intra-arterially with Wittmaack's solution (1911, 1926) so as to fix the tissues *in situ*. It was usual, however in such cases to ligature the femoral artery to one leg before injecting the Wittmaack's solution in order to allow estimation of calcium in the femur of that leg. Part of the liver was also ligatured and removed before the injection took place, in order to allow the estimation of the vitamin A stores. After fixation, blocks of tissue containing the different nerves, together with the adjacent bone, were decalcified by nitric acid and, after embedding in celloidin or paraffin, serial sections were cut and stained with Ehrlich's haematoxylin, Biebrich's scarlet and eosin. By this means it was possible to follow the relations of the bone to the nerve at each point of its course.

Vitamin D_3	10 i.u. daily
Ascorbic acid	5 mg. twice weekly

One or more birds of each hatch were given vitamin A as a supplement to the basal diet. Vitamin A and vitamin D oils were sometimes mixed with the food, but more often were given to the individual birds by pipette.

(f) Adult animals and birds

In some experiments adult animals or birds were used. Their diets were similar in type to those of the young of the same species, but there were not in all cases litter-mates to act as controls.

2. HISTOLOGICAL TECHNIQUE

(a) Nerve fibres

Post mortem changes due to a lapse of time between death of the animal and fixation of its tissues were avoided. In some cases it was found better to fix the tissues *in situ*, but large organs and those with resistant capsules were dissected out and the tissues placed in the fixative.

In connection with the appearance and significance of annular degeneration (see Chapter II), a series of experiments on peripheral nerves was undertaken in order to compare the appearances produced by rough handling in post mortem dissection and those found in experimental animals. Nerves which were crushed with forceps, stretched or left for periods up to forty-eight hours after death of the animal gave positive Marchi reactions, but in these cases the appearances were not the same as those produced by lack of carotene or vitamin A Needless to say, changes produced by trauma were avoided as much as possible.

The tissues were usually fixed in formalin (10 per cent neutralized formol saline)

Although methods have been used for observing changes in the axis cylinders, those for demonstrating degeneration of the myelin sheaths have received most attention. These include: (1) Osmic acid staining by Marchi's method (with modifications [Stewart, 1936]); (2) Nile blue and sulphurous acid (Smith, 1906); (3) Scharlach R.

For peripheral nerves, on the advice of Sir Charles Sherrington, a method described by Ramon-y-Cajal (1928) was also tested in some experiments. Fresh nerves were stained for ten to sixteen hours in 0.5 per cent osmic acid, washed in distilled water, treated

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PART II

THE ANTICALCIFYING OR RACHITOGENIC ACTION OF CEREALS

The Interfering Effect of Cereals on Bone Calcification

Chapter XI

THE DISCOVERY OF THE CEREAL EFFECT

In Part I reference was made to the origin, early in 1915, of an experimental investigation into the aetiology of rickets. The research was made on puppies, and in course of time became directed to the study of nutritional influences on this disease. Brief mention of the discovery of an anti-rachitic fat-soluble vitamin (later called vitamin D), referred to above, was followed by an account of a prolonged investigation, arising directly out of the rickets work, on the action of vitamin A in controlling the shape of bones and on the consequent injury to the nervous system resulting directly from the mis-shapen bones that were formed as the result of the deficiency of this vitamin. It is proposed now to describe and develop another major line of discovery made early in this investigation—namely, the rickets-producing effect of cereals and cereal products.

Prior to the Great War of 1914–18, during which the subject of rickets first became a serious experimental study, among a vast number of other hypotheses it was stated by clinicians that carbohydrate-containing foods, including cereals, had a rickets-producing effect on children. This view was largely founded on the fact that excessive consumption of such foods produced fat babies and in those days fat babies were more prone to develop the disease. Rickets was at that time rampant in both the U. S. A. and Britain and the indictment of this large class of foodstuffs by clinical experts was widely accepted. It will be shown in the following pages that, while their assumption had real foundation, the explanation of the rachitogenic effect of cereals was not so simple as it then appeared, namely that the carbohydrate was the responsible factor.

1. Cereals and Growth Promotion

It was shown (Mellanby 1919, 1920) that, when puppies were brought up on diets deficient in a calcifying vitamin, the degree of rickets became intensified as the consumption of bread increased. The animals which ate most bread put on most weight, and had, within limits, the most rapid growth of bone, and it appeared that a greater intensity of calcification, involving a greater supply of those nutritional agents responsible for bone calcification, was necessary to keep pace with the formation of large amounts of uncalcified bone (osteoid tissue). Bread seemed to be devoid of, or poor in, these calcifying agents, so that more severe rickets developed. This view of the action of bread as a rachitogenic agent involved the basic idea that it acted as a growth-promoter and that rickets was a disease of growth, an idea not far removed from that of the clinicians mentioned above. Experiments were then made to see whether carbohydrate itself in the form of starch and dextrose increased rickets in puppies, but no clear-cut result was obtained as it was not easy to get the animals to eat a diet containing large quantities of pure carbohydrate. These early experiments showed definitely, however, that bread, which promotes growth and at the same time brings with it no compensatory calcifying mechanism, is rachitogenic. It is doubtful, however, whether the starch of bread as such plays much part in increasing the growth of tissues of young animals, although, if other food factors are supplied adequately, increasing the carbohydrate may increase the fat of the animal.

About this time, but from another angle, Hess and Unger (1920) also drew attention to the exceptional power of cereals to promote growth and to cause increase in weight of young animals and infants. They pointed out how frequently infants receiving diets which, according to accepted standards, should be adequate, failed to gain in weight until

cereal was given in addition. For this reason physicians added cereal to the milk diet when there was failure to gain weight about the second half-year of life. Hess and Unger found that, in cases where cod-liver oil no longer caused a gain and egg yolk and beef dripping had failed, a small amount of wheat cereal brought about a decided increase, which could not be due to the simple caloric increase in food, for the amount of cereal added was comparatively insignificant. Cooked cereal equivalent to only 2 or 3 g. of the dry product frequently led to a gain of 2 or 3 oz. by the following day. The findings in regard to rickets and growth promotion in puppies previously mentioned are not, of course, strictly comparable to the observations of Hess and Unger on children, because the amount of cereal fed to the experimental animals formed a substantial part of the diet. From both points of view, however, the action of cereals on growth is emphasized and this undoubtedly is a subject which will repay further study.

2. The Relative Anticalcifying Action of Different Cereals

The direction of research into the rachitogenic action of cereals was altered when it was discovered that, besides having a growth-promoting property, these foods interfered to different degrees with the calcification of the bones of young animals. Thus if a litter of puppies were given the same basal diet devoid of or poor in the antirachitic vitamin, but with a different cereal for each, so that they received equal quantities (by dry weight) of cooked white flour, brown flour, rice, barley or oatmeal, as the case might be, then, although the rate of growth was usually the same, the degree of rickets varied greatly among the animals; the puppy having oatmeal would be much the worst, followed in order by those eating brown flour, barley, rice and white flour (Mellanby, 1922, 1924 and 1925). The results of such an

experiment can be seen in radiographs of the forelegs of a series of puppies (Plate XX a–d). It was obvious that these findings raised a new problem, since some cereals, apart from any growth-promoting quality they had, interfered more with the calcification of young growing bone than others (Exp. 1.)

When these important differences in the production of rickets by various cereals, in the absence or deficiency of the antirachitic vitamin, were first observed, the old idea that carbohydrate was the offending substance in human rickets was still accepted, so it was natural to compare the respective carbohydrate fractions of the cereals with their rachitogenic effect. Oatmeal (the most rachitogenic) has a lower carbohydrate content than either wheaten flour or rice (the respective figures being 65.0, 73.5 and 77 per cent); it was manifest, therefore, that its greater deleterious effect could not be attributed to this moiety.

It may be of interest to recall the views held on the relation of cereals to rickets at the time of these early experiments (Mellanby, 1922). "Cereals, then, (it was stated) increase the laying down of fat and tissues generally, including the bones, and thereby make a greater demand on the calcification processes, so that any tendency to lag behind in this respect is increased by allowing the child or animal to eat a larger quantity of cereal. Increasing the bread, therefore, in a diet which is slightly rickets-producing only emphasizes the disease and causes the development of larger quantities of cartilage and osteoid tissue in the bones. This explanation, however, is not a complete one, for it does not satisfactorily explain the difference between the oatmeal, white flour and rice. In some series of experiments there was no obvious difference between the rates of growth or in the rates of putting on weight when oatmeal, white flour and rice were the only variables, yet the rickets produced by oatmeal was much greater than that produced by

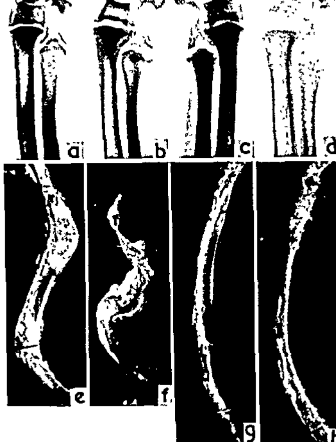


PLATE XX

Experiment 1 a to d. Radiographs showing the different degrees of rickets produced in puppies by diets containing equal amounts of various cereals.

a. Puppy 1 White flour.
b. Puppy 2. Brown flour
c. Puppy 3. Rice.
d Puppy 4. Oatmeal

Note: 1. The severe rickets in d (oatmeal) compared with that in a, b and c (white flour, brown flour and rice).

2 That rickets is worse in b (brown flour) than in a (white flour).

Experiment 2. e to h. Photographs of ribs of four adult dogs whose diets were composed mainly of cereals for a period of 9–10 months.

e and f. Puppies 5 and 6. Corn (maize).
g. Puppy 7. white flour.
h Puppy 8. Rice

Note: The deformity of bone (osteomalacia) produced by corn (maize) and the normal shape of the bones of the dogs eating rice and white flour.

equal quantities of other cereals. Undoubtedly some other factor in oatmeal is at work which either prevents endochondral calcification or increases the formation of tissue at this point, thereby producing a relative lag of calcification at the ends of the bones. It does not appear to be the carbohydrate moiety, and as I have pointed out above, the larger amounts of calcium and phosphorus in oatmeal discredit any explanation which centres round deficiency of these substances. Further experiments will have to be carried out before a definite announcement can be made on this point." It will be seen that it was appreciated, even in those early days of the work, that although much of the cereal action might be explained by the growth-stimulating effect, there was something in the cereal (in this case oatmeal) which had a positive action in interfering with bone calcification.

These early results showing the relative rachitogenic effect of cereals, and especially that of oatmeal, were first announced at a joint discussion on the aetiology of rickets which took place at the Annual Meeting of the British Medical Association at Glasgow in 1922. The late Professor P. Noel Paton and his co-worker, Dr. Leonard Findlay, working in Glasgow were unable to accept the foregoing views, based on experiments on puppies, that diet was the dominant factor in the aetiology of rickets, and more especially that an antirachitic fat-soluble vitamin was the key to the situation and the essential dietetic factor preventing the disease. They were protagonists of the view that rickets was due to defective hygiene and more particularly to those conditions, such as bad housing, which they thought limited exercise of infants and children. They also based their views largely on experiments on puppies (Findlay, 1908), which they said demonstrated that confinement and lack of exercise brought about earlier and more severe rickets and that freedom to exercise either prevented or delayed the

development of this condition. These results, they believed, were in keeping with the higher incidence of rickets in children in cases of overcrowding, as revealed by a series of social and medical surveys (Ferguson, 1918). Those present at the discussions during that period will remember the intense controversial feeling with which they were pervaded. It can therefore be imagined how the new indictment at Glasgow of the Scottish National food, oatmeal, as the king of rickets-producers roused much emotion, among both those participating in the research and the onlookers. Matters were not improved by the knowledge that the incidence of rickets in Glasgow at the time was probably higher than in any other part of the world. Nor did the matter stop at rickets, for it was shown by M. Mellanby (1923 and 1929) that, with a deficiency of the antirachitic vitamin, oatmeal also produced in young growing animals teeth of worse structure than any other cereal tested. The indignation in Scotland was not only due to the so-called "attack" on their national food, but also to the undoubted fact that in years past, when oatmeal was their staple diet, the Scots were known to have been one of the best grown and lustiest nations of the world, with particularly fine bones and teeth. The answer to this enigmatical situation was, of course, that when oatmeal was eaten in such large quantities, milk was the other constituent of the diet among the agricultural community, while among the fishermen, fish, including the liver, was consumed on a large scale. In fact, there was really no problem at all, for the experimental evidence put forward by the author showed that a high antirachitic vitamin and high calcium intake could antagonise and overcome any anticalcifying effect of cereals. The practical lesson was that, if cereals formed a large proportion of the diet, and especially if oatmeal was the main cereal, good nutrition could only be obtained by an adequate fat-soluble vitamin intake, together with

abundant calcium, such as is obtained in a diet rich in milk.

Some idea of the popular interest and deep feeling caused in Scotland by the discovery of the rickets-producing action of cereals, which, it may be added, was not for many years accepted as true or considered even possible (see page 9), can be seen in Fig. 42, which is a cartoon produced in a



ONE MANS MEAT. IS ANOTHER DOGS POISON!

Fig. 42. Cartoon reproduced from a Glasgow Newspaper, published in 1922

This cartoon was published when the rickets-producing effect of oatmeal, *the Scottish national food*, was first described.

Glasgow newspaper during the time of the meeting in that city in 1922. It is easy now to look back on those days with equanimity, for the rachitic action of cereals, and especially of oatmeal and corn (maize), is generally accepted and, what is much more important, rickets has been practically abolished from Glasgow and most other places in Scotland and England *by the adoption of the methods then advocated.*

Following briefly the history of this subject, it may be noted that by 1924 this question of the cereal effect on bones had crystallised out. In a lecture given in that year (Mellanby, 1924) it was stated: "The rickets-producing effect of cereals appears to be best explained by assuming that there is in cereals a substance or substances actively preventing bone calcification. This substance, whose composition and properties are unknown, appears to act in a diametrically opposite way to the antirachitic vitamin."

Note: Before passing on to the main problem, it must be mentioned that by 1922 Huldschinsky's observations on the therapeutic effect of ultra-violet light on rickets had become widely known and accepted both in the U.S.A. and in England (Huldschinsky, 1919, 1920). The general interest and widespread investigations which followed, in order to link up the action of the antirachitic vitamin of food and the antirachitic effect of ultra-violet radiations, were remarkable. This is an old story and will not be discussed here, but probably it will not be out of place to mention a few of the outstanding results which led to the elucidation of the problem, viz. the relation of the antirachitic action of the fat-soluble vitamin D in the diet, and the antirachitic effect of ultra-violet radiation striking the skin.

(a) The effect of the antirachitic vitamin on bone calcification was confirmed by experiments on rats (Shipley, Parke, McCollum, Simmonds and Parsons, 1921; Korenchevsky, 1922; Goldblatt, 1923)

(b) Up to a certain point ultra-violet radiations acting on the skin could replace cod-liver oil in its promotion of the growth of young rats (Hume, 1922; Goldblatt and Soames, 1923).

(c) The livers of irradiated rats given a fat-soluble vitamin-free diet acquired growth-promoting properties, while those of control, non-irradiated rats were inactive (Goldblatt and Soames, 1923).

(d) The irradiation of certain foods, themselves incapable of promoting growth, when added to diets deficient in fat-soluble vitamins, conferred upon them growth-promoting and bone-calcifying properties, e.g. olive, cotton seed and peanut oil (Steenbock and Black, 1924)

(e) By the irradiation of what, at the time, were thought to be pure sterols—cholesterol, and phytosterol—growth promoting and

calcifying properties were produced (Steenbock and Black 1925; Hess, Weinstock and Helman, 1925; Rosenheim and Webster, 1925).

(f) It was later shown that it was not cholesterol itself but an impurity, ergosterol, which was activated by ultra-violet radiations (Rosenheim and Webster, 1926, 1927; Windaus and Hess, 1927).

It will now be shown how these investigations, carried out on an international scale, on the relation of ultra-violet radiation to the antirachitic vitamin D, affected the cereal researches under discussion. It seemed of interest to see the effect of ultra-violet radiation on (1) the rachitogenic action of oatmeal, and (2) puppies eating diets which were actively rachitogenic because of the absence of the calcifying vitamin and the presence of much non-irradiated oatmeal. As regards (1), it was shown that exposing oatmeal to the radiation of a mercury vapour lamp antagonised its rickets-producing effect, so that dogs eating the treated cereal remained completely free from rickets while litter mates on diets of the same type but containing non-irradiated oatmeal developed the disease. However if this irradiated cereal was fat-extracted, the antirachitic factor was dissolved out by the solvent and the residue was as intensely rickets-producing as the non-irradiated cereal. It was clear that subjecting oatmeal to ultra-violet rays had converted its ergosterol to vitamin D, which had cloaked the action but had not destroyed the rachitogenic factor, since it remained after the removal of the vitamin D by ether extraction (Mellanby, 1925).

When the radiation of the mercury vapour lamp or sunlight of sufficient intensity was allowed to fall on the animals, it was soon evident, as expected, that an antirachitic influence was being exerted. It was apparent that, in tropical countries, where cereals such as rice, maize and millet formed a large part of the diet, the sunlight acting on large areas of the skin of unclothed people, was a most important

factor in antagonising the detrimental influence of the diet, and it still remains so in districts which have not adopted 'civilized' customs in regard to clothing and food. It was in countries like Britain where the average diet was deficient in, or contained a border-line quantity of, antirachitic vitamin and calcium (because of the relatively low intake of milk, cheese and eggs) and where sunshine or at least its ultra-violet component was negligible, and the temperature of the atmosphere made it essential to cover the body with clothes, that the ingestion of large quantities of cereal by women during pregnancy and lactation and by growing children did much harm. It will be seen in the final chapter (page 423), however, that in recent years there has been a great improvement in the nutritional value of the British diet, especially from the point of view under discussion.

Before leaving this subject of the antirachitic effect of ultra-violet radiations, it is necessary to say that, at this time, it was assumed that the vitamin produced by irradiating fats (in particular their ergosterol fraction) and the skin was the same substance as is found naturally in animal fats especially of fish. We know now that this is not the case but that there are a number of antirachitic vitamins, the most important of which are vitamins D_2 and D_3; that D_2 is produced by irradiating ergosterol with ultra-violet radiations and D_3 by irradiating 7-dehydrocholesterol. How complicated the subject now is can be seen from the fact that cod-liver oil is said to contain, on the basis of molecular distillation experiments, six forms of vitamin D.

In 1925 a more complete account of the action of cereals in producing rickets was published (Mellanby). By this time it was found that maize (Indian corn) next to oatmeal, had a powerful rachitogenic effect—a fact which Steenbock, Black and Thomas (1930) later emphasised; indeed they thought that corn was worse than oatmeal;* wheat germ

* *Note:* As cereals of any type appear to vary in their rachitogenic activity some corns may even be more rachitogenic than some oatmeals.

calcifying properties were produced (Steenbock and Black 1925; Hess, Weinstock and Helman, 1925; Rosenheim and Webster, 1925).

(f) It was later shown that it was not cholesterol itself but an impurity, ergosterol, which was activated by ultra-violet radiations (Rosenheim and Webster, 1926, 1927; Windaus and Hess, 1927).

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Attention was then directed to the Ca:P ratio of the cereals, since work on rickets in rats had indicated that this was an important factor when the calcifying vitamin was deficient in the diet (McCollum, Simmonds, Shipley and Park, 1921). However, examination of these ratios did not seem to advance the investigation. Thus oatmeal and white flour, at opposite extremes in their rachitogenic action, were found to have calcium-phosphorus ratios of the same order, i.e., 0.175:1 and 0.217:1 respectively, whereas rice and wheat germ, also differing in their effect on bone calcification, had the much lower ratios of 0.094:1 and 0.068:1 respectively. The complete discordance between the Ca:P ratios, the amounts of these elements in the cereals examined, and their rachitogenic action seemed, at the time, to preclude further investigation into this aspect of the subject (Mellanby, 1925). It was also written: "Since the antirachitic vitamin supplies in the food and exposure of the animal or the food eaten to ultra-violet radiations tend to conserve ingested calcium and phosphorus for the use of growing organism, and since the cereals work in the opposite direction, it is evident that the amount of calcium and phosphorus in the food is of but secondary importance in the control of the deposition of these elements in growing bone, although, of course, there must be a sufficiency of these salts available for the formation of perfect bones. In view of the evidence of interaction and balance among food constituents provided by this investigation, the value of the expression 'optimum calcium content of a diet,' so commonly used in dietetic descriptions and discussions nowadays, must be doubted. The optimum varies every time the other elements of diet are changed." These words written 22 years ago are still true and their significance, in practice at least, almost as unappreciated as it was then, in spite of the large amount of nutritional research carried out all over the world in the intervening years.

was also implicated and bread made from high extraction flour was found to produce rather worse rickets than white flour. Another result which became of greater significance in later years was that, even when there was a deficiency of antirachitic vitamin (see page 336), the addition of calcium carbonate, calcium phosphate (bone ash) or calcium acid phosphate hindered to some extent the development of rickets (Mellanby, 1925). It was found that, under the experimental conditions, calcium carbonate was rather more effective in delaying rickets than calcium phosphate. On the general problem of the rickets-producing effect of cereals it was now stated that two different kinds of action appeared to be at work. In the first place that part of the cereal which was actually incorporated in the growing organism and led to its growth was probably partially responsible. In this way the carbohydrate and the protein would be involved. In the second place (it was stated), there was present in the one cereal tested, namely oatmeal, a chemical grouping which, after digestion was capable of interfering with bone calcification. Attempts were made to isolate this unknown body and one experiment was described which, at the time, seemed to indicate that the rachitogenic substance could be obtained by extracting with ether oatmeal which had been boiled with soda. It is now known that this deduction was not true.

Almost as soon as the varying rachitogenic effect of different cereals had been discovered, the possibility was considered that the calcium and phosphorus contents of these substances were in some way implicated in this action. It was at once clear that the absolute amounts of calcium and phosphorus could not explain the effect, since those cereals which contained most of these elements such as oatmeal and corn, were also the most rachitogenic, whereas rice and white flour, interfering least with bone calcification, contained the least calcium and phosphorus.

other hand, when white flour and rice were the cereals eaten, even although the calcium and vitamin D contents of the diets were very low, and indeed the calcium was lower than in the oatmeal and corn-fed animals, the bones were better calcified and contained but little osteoid tissue. In other words, deficiency of calcium and vitamin D alone did not seem to be sufficient to produce osteomalacia; there had to be some predatory decalcifying quality of the cereal further hastening the loss of calcium from the bones. The relative effect of these cereals, as illustrated by bone deformity, can be seen in Plate XX e—h, which consists of photographs of the costochondral junctions of four dogs, two having been fed on a diet rich in Indian corn (maize) and two on diets similar in other respects but rich in white flour and rice. The deformity of the ribs of the corn-eating dogs is in contrast to the normal shape of the ribs of the flour and rice-eating animals (Exp. 2). When either cod-liver oil, calcium carbonate or calcium phosphate was added to the diets, even when corn and oatmeal were the cereals eaten, no deformity of the bones developed in these experiments.

4. The Presence of a Specific Anticalcifying Substance in Cereals

The experiments referred to above leave no doubt that the action of cereals in interfering with calcification varies, being more potent in some than in others, that it influences both the deposition of calcium salts in growing bone and the decalcification of adult bone, and that it is dependent on some property of the cereals which renders calcium of the diet unavailable to the animals or even deprives them of the stores in their own bodies.

In 1926 (Mellanby) another kind of evidence came to light, emphasising this aggressive action of cereals against calcium salts in the food and in the body and further supporting the view that it was due to a specific chemical

3. Withdrawal of Calcium from the Bones of Adult Animals

Up to this point the action of cereals has been demonstrated and discussed only in relation to the calcification of bones of young growing animals, and indeed almost entirely of puppies. It seemed of interest to extend the investigation and to find out whether those cereals which most actively prevented the deposition of calcium salts in growing bones could also exert a decalcifying action on fully calcified bones,—whether, in fact, they could hasten the development in adult healthy dogs of osteoporosis and osteomalacia. The problem was now one of the withdrawal of calcium salts already present in the bones, in contrast to the earlier experiments on young dogs, where the study was of factors affecting the deposition of calcium salts in bone.

A brief account of the results of experiments made to decide this question will now be given. Adult dogs were fed on diets very rich in cereals and deficient in vitamin D. Sometimes the cereals were those which had been shown by the earlier work to have a powerful rachitogenic effect, such as oatmeal and corn, and sometimes the cereals chosen had the least anticalcifying action, such as white flour and rice. In addition to the absence or deficiency of vitamin D, the calcium contents of the diet were kept low. Meat which had been carefully cleaned of fat also formed part of the diet, together with a vegetable oil, such as peanut oil. These adult animals were kept on diets of this nature for periods varying from 7–12 months. Bone deformity was only produced in the animals whose diets included large quantities of those cereals with powerful anticalcifying action , i.e. the corn and the oatmeal dogs. The bones of the rice and white flour animals were osteoporotic but showed no deformity. The corn-eating dogs had bones with much osteoid-like tissue and a very low calcium content and somewhat similar bones were produced in those having oatmeal. On the

the presence of the cereal factor when vitamin D was deficient, were more thoroughly absorbed and incorporated in bones and teeth of growing puppies when the vitamin was present in sufficient quantity. It was this property of antagonising calcium retention which suggested the name anticalcifying toxamin for the unknown substance in cereals. In the next Chapter will be seen the importance of these preliminary observations on acid hydrolysis and malting in tracking down this substance.

component of these foodstuffs. It was found that a powerful anticalcifying cereal like oats (or oatmeal) lost some of its action after certain simple forms of chemical treatment, namely, (1) boiling with dilute hydrochloric acid, and (2) malting, i.e. germination followed by heating. Since neither of these forms of treatment could alter either the total carbohydrate of the cereal or the total or relative amounts of calcium and phosphorus, it was considered that the new evidence strongly supported the idea of a positive anticalcifying agent, the action of which could be reduced by suitable simple treatment which probably involved hydrolysis. It was not contemplated at the time, however, that these forms of treatment might alter the availability to the body of the calcium and phosphorus of the cereals.

Although these results revived the problem as an experimental study, it will be seen later (see page 284) that both forms of treatment of cereals required much further investigation (after their preliminary publication) before they were fully understood and their action could be effectively controlled. However, these crude experiments increased the probability that cereals contained a definite anticalcifying substance whose action could now be modified in two entirely different ways, (1) by adding vitamin D to the diet, and to a less extent by adding calcium salts, and (2) by destroying the hypothetical toxic substance by boiling with acid and also by malting and other treatment following germination. These qualities brought about the introduction of the word 'toxamin' to describe a harmful food substance whose action could be antagonised by a vitamin (see Part I, page 65). The rickets-producing effect of cereals was wholly or partially due to the presence of such a toxamin, whose composition was unknown (Mellanby, 1937). The calcium and phosphorus of the diet, and especially the calcium, which were lost to the body in

in availability. The original rachitic state was produced by feeding rats on a high Ca-low P diet deficient in vitamin D, and it was found that phytin (Ca Mg inositolhexaphosphate) or sodium phytate caused only a small degree of healing, while the addition of sodium hydrogen phosphate, having the same content of P, was more effective in this respect. Lowe and Steenbock (1936 a) found that the proportion of Ca in the diet of rats was one of the factors affecting the availability of the phytin P, and that this was greatly diminished by the addition of $CaCO_3$ to a diet of low Ca content.

The relative unavailability of phytic acid P was found to hold also for human beings by McCance and Widdowson (1935), who determined the actual amount of this substance in the faeces after the addition of phytin to the diet. They found that 20 to 60 per cent of the phytin was excreted unchanged in the faeces and suggested that much of the remaining phytin P may also have been broken down by the intestinal flora at a level below that at which absorption could take place.

The above investigations drew the attention of the author to the possibility that phytic acid was the rachitogenic toxamin in cereals which had been sought since 1926. Not that the results of the rat experiments led to this hypothesis, since the point established by them was that the phosphorus of phytate was available only with difficulty to the animal and that this difficulty was increased by the addition of more $CaCO_3$. In the puppy experiments, on the other hand, the main characteristic of the anticalcifying substance in cereals was that it prevented the use and availability of calcium and had a positive rickets-producing effect. There were, however, some other qualities possessed by phytate which indicated that it might well be the elusive factor in cereals. For instance, phytate could be hydrolysed to inositol and phosphoric acid by acid and also by the

Chapter XII

THE IDENTIFICATION OF THE ANTICALCIFYING SUBSTANCE IN CEREALS

1. Preliminary Observations

It has long been known that a large part of the phosphorus compounds in cereals is in organic combination, that much of this organic phosphorus is in the form of phytic acid (inositol hexaphosphoric acid) and that this is present, in part at least, as the Ca Mg salt which is known as phytin.

Some of the earliest work on the physiological action of phytin by Starkenstein (1910) and Plimmer (1913) provided evidence that this substance, when fed to animals, was not directly absorbed from the alimentary canal and that such breakdown of the compound as occurred in the intestine (leading to the formation of inositol and phosphoric acid) was probably largely the result of bacterial action. The possible hydrolysis of phytin by intestinal bacteria will be considered later (see page 388).

Steenbock, Black and Thomas (1930) drew attention to the possibility that inorganic phosphate added to the diet may not be equivalent in physiological properties to the organic phosphorus compounds in cereals and confirmed the earlier work of Starkenstein and Plimmer referred to above.

In 1934 Bruce and Callow, following up the deduction from the work of Steenbock, Black and Thomas (1930) that cereals contain a form of phosphorus less available to animals than inorganic phosphate, showed that the phosphorus of phytin was much less available to rats than that of sodium phosphate and that the relative curative effect of these substances on rickets, when given with small quantities of vitamin D, was dependent on this difference

however, that under such conditions, the action of phytin might be different and drew attention to the work of Starkenstein (1914) on the possible action of phytic acid in precipitating calcium or rendering it un-ionized.

Such a possibility seemed much more in accordance with the observations of the author, particularly with the fact that adding calcium to the diet reduced the rachitogenic effect of the cereals. Indeed, with this possibility in mind, experiments were made to isolate the rickets-producing factor of the HCl extracts of oatmeal by precipitation of the neutralized filtered extract with $CaCl_2$. On feeding the product, after removal of Ca as oxalate, no definite result was however, obtained, probably because the substance was given in insufficient amounts. An observation which also encouraged the view that phytic acid was in some way concerned with the cereal action was the fact that Holst (1927) had shown that the active factor could be extracted from oatmeal by cold dilute HCl, for earlier workers had used this method for extracting phytic acid from foodstuffs prior to its estimation by iron titration (Heubner and Stadler, 1914).

Altogether it seemed desirable to test the effect of feeding phytic acid and phytin to puppies, using diets with a Ca:P ratio of the type frequently met in human, and especially children's diets, and not that used in the rat experiments. This was the object of the animal and other experimental work described in this chapter, which was carried out in collaboration with Dr. D. C. Harrison, now Professor of Biochemistry at Belfast University (Harrison and Mellanby, 1939). If phytic acid showed a rachitogenic action, and, if this action were due to interference with calcium absorption by precipitation of the base, it would be expected that in such experiments, unlike those with a high Ca-low P ratio, phytin, the CaMg salt, would show little or no rachitogenic action.

phytase present in cereals. Therefore it might well be the substance in cereals whose anticalcifying effect, as has been mentioned in Chapter XI, could be reduced by boiling in mineral acids or by allowing grains to undergo malting changes.

Further consideration, however, only emphasized the fact that these various experiments on the relative availability of phosphorus in different compounds had but little effect on the rickets-producing action of cereals described in Chapter XI. Quite apart from the phytin P, there was in the diets sufficient phosphorus in other forms to produce good bones in the experimental puppies; moreover, unlike Bruce and Callow and also Steenbock, Mellanby (1925) had found that, under the experimental conditions, the addition of sodium phosphate to the diet of puppies produced no improvement in calcification. The high Ca-low P diet (at least Ca:P 4:1) used by the above mentioned workers to produce rickets in rats was not the type of diet which produced rickets in children, a condition that could normally never be improved, much less cured, by adding inorganic phosphate to the diet. Indeed, from the point of view of its relation to human nutrition, the high Ca-low P diet was an artificial one. The dietary conditions in the experiments on puppies approached more closely those which led to rickets in children.

The point at issue was clearly, not whether phytin P was available, but whether phytin in cereals was responsible for their positive rickets-producing effect under ordinary dietetic conditions. Bruce and Callow themselves pointed out that their interpretation of the phytin effect as being due to the relative unavailability of its phosphorus was based on results obtained with the high Ca-low P diet used in studying rickets in rats, and that they had attempted to extend their observations to low Ca-high P diets, but definite results had not been obtained. They suggested,

time such animals may not eat their full ration and consequently their increases in weight may be slower than the rest of the litter. When this occurs the experiment is usually ended.

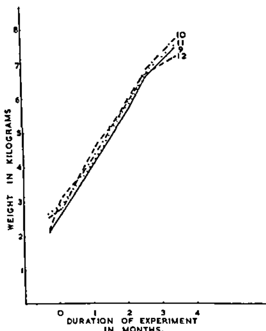


FIG. 43 (Exp. 3). Weight increases of a litter of 4 puppies.
These weight curves are typical of those of the litters of experimental puppies described in this chapter. (For variables in diet of above animals see Experiment 3, p. 230.)

There is one other special point about these phytic acid experiments worthy of mention. In order to test the effects of some substance which might increase the degree of rickets, it is necessary to make the control diets of such a nature that rickets is either produced only slightly or just prevented by a narrow margin. If, for instance, the control animals develop severe rickets, the effect of adding a rickets-

It is realised that in these early experiments the total phosphorus of the diets of the animals was not entirely controlled, but in the later work (from Experiment 7 onwards) unless otherwise arranged this factor was kept constant and shown to be relatively unimportant from the point of view of the main problem.

2. Experimental Work on Phytic Acid in Relation to Rickets

The experimental technique used was similar to that of earlier work on rickets and the severity of the disease was appraised by (1) the appearance of the dog, including the degree of leg deformity and the size of the epiphyseal ends of the bones and of the costochondral junctions; (2) the radiographic appearance of the epiphyseal ends of the bones; (3) the degree of bone calcification as determined by ash and Ca content; and (4) the histological appearance of the bones. As regards the degree of calcification, the measure used was the A/R ratio, when A is the weight of ash of the bone and R that of the dry fat-extracted bone minus the weight of the ash (Chick and Roscoe, 1926; Chick, Korenchevsky and Roscoe, 1926). This ratio was devised to overcome the difficulty caused by the great variation in the amount of marrow fat in the bones of these experimental animals, a variation which prevented the easy correlation of calcium and the total weight of bones of rachitic and normal animals.

In view of the close association discussed above between rickets and growth, it is essential in such work, when comparisons are to be made, to adjust the amount of food so that all the animals in a litter eat equal amounts of the basal diet, so that their growth (as measured by weight increase) is as similar as possible (Fig. 43). This object is usually easily attained up to the point when some member or members of the experimental litter develop severe rickets. After this

Age at beginning of experiment: 8 weeks.
Duration of experiment: 13½ weeks.

TABLE 4 (Exp. 3)

Relative effects on bone calcification of phytin and sodium phytate

No. of puppy	Additions to basal diet			Bone results	
	Phytin	Na phytate	Calcium lactate	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.*
9	None	None	None	1.08	5
10	0.6% of cereal	None	None	1.33	3
11	None	Amt. ≡ phytin 0.6% of cereal†	None	0.76	7
12	None	Amt. ≡ phytin 0 6% of cereal†	0.554% of cereal	1.39	3

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

† For preparation of Na phytate used in this experiment see Harrison and Mellanby, 1939, p. 1664.

It will be seen that in this experiment, phytin added daily to the basal diet to the amount of 0.6 g., increasing to 0.9 g., had some antirachitic influence and actually improved the calcification of puppy 10 as compared with 9. On the other hand, the addition of sodium phytate equivalent to 0.6 to 0.9 g. of phytin increased the rickets of 11 and greatly diminished the calcium in the bones. The addition of Ca to the diet of 12 in the form of calcium lactate abolished the rachitogenic action of the sodium phytate and made it similar in action to phytin.

Experiment 4

The object of this experiment was to test the effect on bone development of phytic acid and neutral sodium

producing substance to the diet of other animals of the litter may be lost because all animals are so severely affected. If, on the other hand, the basal diet is too antirachitic, the effect of the rachitogenic substance to be tested may be so far overcome as to escape detection by X-rays.

Experiment 8

Object. This experiment was made to test the relative effects of phytin and sodium phytate on the development of the bones of puppies of the same litter.

Basal experimental diet:

White flour	100–150 g.
Separated milk powder	15– 26 g.
Lean meat	15– 22.5 g.
Orange juice	6 ml.
Yeast	5– 7.5 g.
NaCl	2 g.
Peanut oil	10 ml.
Cabbage	20 g.

(Changes in amount of food ingredients in this and other experiments represent increases in intake with advancing ages of puppies.)

Daily additions to basal diet

No. of puppy*	Phytin	Na phytate	Calcium lactate
9 (2122)	None	None	None
10 (2123)	0.6% of cereal	None	None
11 (2124)	None	Amt. ≡ phytin 6% of cereal	None
12 (2121)	None	Amt. ≡ phytin 6% of cereal	0 554% of cereal

* The numbers in parentheses in this and other experiments are the actual serial numbers of the experimental animals.

Age at beginning of experiment: 8 weeks.
Duration of experiment: 13½ weeks.

TABLE 4 (Exp 3)

Relative effects on bone calcification of phytin and sodium phytate

No. of puppy	Additions to basal diet			Bone results	
	Phytin	Na phytate	Calcium lactate	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.*
9	None	None	None	1.08	5
10	0.6% of cereal	None	None	1.33	3
11	None	Amt. ≡ phytin 0.6% of cereal†	None	0.76	7
12	None	Amt ≡ phytin 0.6% of cereal†	0.554% of cereal	1.39	3

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

† For preparation of Na phytate used in this experiment see Harrison and Mellanby, 1939, p. 1664.

It will be seen that in this experiment, phytin added daily to the basal diet to the amount of 0.6 g., increasing to 0.9 g., had some antirachitic influence and actually improved the calcification of puppy 10 as compared with 9. On the other hand, the addition of sodium phytate equivalent to 0.6 to 0.9 g. of phytin increased the rickets of 11 and greatly diminished the calcium in the bones. The addition of Ca to the diet of 12 in the form of calcium lactate abolished the rachitogenic action of the sodium phytate and made it similar in action to phytin.

Experiment 4

The object of this experiment was to test the effect on bone development of phytic acid and neutral sodium

phytate, both of which were made from a commercial preparation of phytin by a method based on that of Posternak (1921) (see Appendix II, p. 433).

Basal experimental diet:

White flour	110–150 g.
Separated milk powder	20– 25 g.
Lean meat	15– 20 g.
Cabbage	10– 20 g.
Baker's yeast	5– 7.5 g.
Peanut oil	15 ml.
Orange juice	6 ml.

Variations in and daily additions to basal diet:

No. of puppy	
13 (2517)	None.
14 (2515)	Oatmeal replaced white flour of basal diet.
15 (2518)	Sodium phytate* (neutral) added.
16 (2519)	Phytic acid* added.

* See footnote to Table 5.

Age at beginning of experiment: 10 weeks.

Duration of experiment: 7½ weeks.

Both sodium phytate (neutral) and phytic acid had rickets-producing effects of about the same degree of intensity, but they were not quite so powerful in this respect as oatmeal, although the amount of phytic acid or phytate added was equivalent, to the amount present in the oatmeal eaten by puppy 14.

Having established from the foregoing and other experiments that the rachitogenic action of oatmeal in the diet could be largely imitated by feeding phytic acid or neutral sodium phytate (alkaline sodium phytate was less potent), it seemed reasonable to attempt an isolation of the rickets-producing agent by working on the assumption that the

cereal factor might be phytic acid or some chemically similar compound. This assumption seemed a likely one in view of the fact that cereals are known to be rich in phytin P.

The first step in such an attempt was to get the oatmeal phytate (oatate) into solution. Extraction with dilute HCl

TABLE 5 (Exp. 4)

Effects on bone calcification of phytic acid and sodium phytate (neutral)

No of puppy	Dietary conditions: Type of cereal	Dietary conditions: Additions to basal diet	Bone results: A/R ratio of femur shaft	Bone results: Rickets as judged by X-rays at P.M
13	White flour	None	0.97	5
14	Oatmeal	None	0.84	9
15	White flour	Sodium phytate* (neutral)	0.86	7
16	White flour	Phytic acid*	0.85	8

Rickets graded 1 to 10, the number increasing with the severity of the disease

* The phytic acid phosphorus added to the diets of animals 15 and 16 was approximately equal to that of the oatmeal consumed by puppy 14 The oatmeal contained 0.225% phytic acid phosphorus, and the actual amount of phytic acid phosphorus in the diet of the animal receiving oatmeal increased from 247 5 mg to 337.5 mg. during the experiment.

seemed the obvious choice, for there was already evidence that the rachitogenic factor could be extracted from oatmeal with HCl (Holst, 1927; de Bruin and Bouman, 1937), but it was at first found impossible to obtain an extract which was filterable, unless so large a volume of HCl was used relative to the amount of oatmeal that the preparation became too

bulky to handle on the large scale necessary for feeding dogs. The difficulty was finally overcome by first defatting the oatmeal, treating with diastase to break down most of the starch, and then extracting with HCl. In this way it became possible to obtain clear HCl filtrates using reasonably small volumes of fluid. (*For further details as to the method used* see Harrison and Mellanby, 1939, p. 1669.)

The crude salt prepared from oatmeal (exp. 5) was first referred to as sodium oatate. Several experiments, of which the following is an example, were carried out with this oatate and sodium phytate prepared from phytin.

Experiment 5

The object of this experiment was to see whether sodium oatate prepared from oatmeal had the same effect on calcification as sodium phytate of equal P content prepared from phytin.

Basal experimental diet:

White flour	52–254 g.
Separated milk powder	20 g.
Lean meat	8– 38 g.
Baker's yeast	2.4– 11.4 g.
Cabbage	11.2– 53.2 g.
Peanut oil	6– 28.5 ml.
NaCl	0.8– 3.8 g.
Orange juice	6 ml.

Variations in and daily additions to basal diet:

No of puppy	
17 (2643)	None.
18 (2641)	Oatmeal replaced half of white flour of basal diet.
19 (2642)	Sodium phytate* (from commercial phytin) added.
20 (2644)	Sodium oatate* (from oatmeal) added.

* See footnote to Table 6.

Age at beginning of experiment: 7½ weeks.
Duration of experiment: 16 weeks.

TABLE 6 (Exp. 5)
Effect on bone calcification of neutral sodium phytate prepared from phytin and from oatmeal

No of puppy	Dietary conditions		Bone results	
	Type of cereal	Additions to basal diet	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M
17	White flour	None	1.36	2
18	50% white flour, 50% oatmeal	None	1.11	5
19	White flour	Sodium phytate* (from commercial phytin)	1.02	4
20	White flour	Sodium oatate* (phytate from oatmeal)	1.16	6

Rickets graded 1 to 10, the number increasing with the severity of the disease.

* The phytic acid phosphorus added to the diets of animals 19 and 20 was approximately equal to that in the oatmeal consumed by puppy 18 The oatmeal contained 0.290% phytic acid phosphorus, and the actual amount of this substance in the diet of the animal receiving oatmeal increased from 75 5 mg. to 368 mg. during the experiment.

In this experiment phytic acid phosphorus, whether given as sodium phytate prepared from commercial phytin or as sodium oatate prepared from oatmeal, increased the degree of rickets and slightly reduced the calcium in the femur shafts. The anticalcifying effect of these two substances appeared to be approximately equal, and of the same order as that produced by substituting half the white flour of the basal diet by oatmeal.

Although the biological results suggested that the sodium oatate was in fact sodium phytate it was decided to undertake further purification of this salt. Analysis of a recrystallized air-dried alkaline salt (Harrison and Mellanby, 1939, p. 1672) gave the following results:

Loss of water after drying over H_2SO_4 and then at 120°	= 38.42%
Ash after ignition	= 50.69%

The hydrated salt melted at 56–59°, the melting point not being sharp.

According to Posternak (1921), the air-dried alkaline sodium phytate has the composition $C_6H_6O_{24}P_6Na_{12}$, $3H_2O$ + $35H_2O$, and melts at 58–59°.

Calculated loss for $35H_2O$ = 39.18%
Calculated ash ($3Na_4P_2O_7$ = 49.63%

On titration with N/10 HCl to a faint rose colour with methyl orange, 5.96 equivalents of acid were required. (Posternak found six equivalents.)

The salt crystallizing with $44H_2O$ described by Posternak was also prepared by allowing the solution to crystallize at about 2°, filtering on an ice-cold funnel, washing with cold water and drying on an ice-cold porous tile. This salt readily redissolves in the mother liquor if allowed to warm up to room *temperature before it is dry.*

While, therefore, the preparation obtained from oatmeal is not quite pure, it accords closely in composition and properties with sodium phytate as described by Posternak (1921).

It would have been desirable to have used the recrystallised phytate, as prepared from oatmeal for chemical analysis, in the feeding experiments, but the losses were so great that this was not found possible, and in most experi-

ments the purified neutral sodium salt extracted from oatmeal and precipitated by alcohol was used.

Experiment 6

The object of this experiment was to test the effect on bone development of purified neutral sodium phytate prepared from oatmeal.

Basal experimental diet:

White flour	64.5–90.3 g.
Separated milk powder	20.0 g.
Lean meat	10–14.0 g.
Cabbage	14.0–19.6 g.
Baker's yeast	3.0– 4.2 g.
NaCl	1.0– 1.4 g.
Peanut oil	7.5–10.5 g.
Orange juice	6.0 ml.

Variations in and daily additions to basal diet:

No of puppy	
21 (2703)	None.
22 (2706)	None.
23 (2702)	Defatted oatmeal replaced white flour of basal diet.
24 (2701)	Sodium phytate (from oatmeal)* added.
25 (2705)	Sodium phytate (from oatmeal)* added.

* See footnote to Table 7.

Age at beginning of experiment: $7\frac{1}{2}$ weeks.
Duration of experiment: $10\frac{1}{2}$ weeks.

The sodium phytate eaten by puppies 24 and 25 produced a severe degree of rickets, which is seen from the appearance of the animals (Plate XXI, i and j), the radiographs (Plate XXI, d and e) and the A/R ratios of the femur shafts. The degree of rickets in these two puppies is comparable with that produced in 23 by the diet containing defatted oatmeal.

The foregoing experiments lead to the following main conclusions: When fed to dogs receiving basal diets of the type described, (a) commercial phytin (CaMg phytate) is not rachitogenic; in fact, it is, if anything slightly antirachitic; (b) sodium phytate or phytic acid prepared from

TABLE 7 (Exp. 6)

Effect on bone calcification of sodium phytate

No. of puppy	Dietary conditions		Bone results	
	Type of cereal	Additions to basal diet	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.
21	White flour	None	1.20	3
22	White flour	None	1.17	5
23	Defatted oatmeal	None	0.97	9
24	White flour	Sodium phytate* (from oatmeal)	0.87	9
25	White flour	Sodium phytate* (from oatmeal)	0.97	10

Rickets graded 1 to 10, the number increasing with the severity of the disease.

* The sodium phytate was prepared from the same batch of defatted oatmeal as that used for puppy 23, and the phytic acid phosphorus added to the diets of animals 24 and 25 was approximately equal to that of the oatmeal consumed by puppy 23. The oatmeal contained 0.335% phytic acid phosphorus and the actual amount of this substance in the diet of puppy 23 increased during the experiment from 216 mg. to 302 mg. per day.

a commercial phytin is definitely rachitogenic, being comparable in potency with oatmeal when fed in a quantity equivalent to the total phytate of the cereal; (c) sodium phytate prepared from oatmeal retains approximately the full rachitogenic activity of the oatmeal, and this activity is not diminished by further purification;

(d) a sufficient amount of Ca counteracts the rachitogenic activity of sodium phytate, as it does that of cereals.

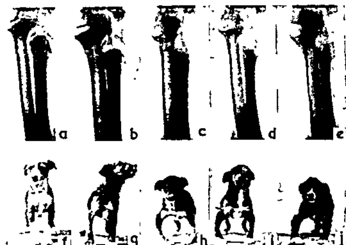


PLATE XXI

(*Experiment 6.*) Radiographs of paws and photographs of puppies showing the effect of sodium phytate on bone calcification.
a and f. Puppy 21. White flour.
b and g. Puppy 22. White flour.
c and h. Puppy 23. Oatmeal.
d and i. Puppy 24. White flour and Na phytate prepared from oatmeal.
e and j. Puppy 25. White flour and Na phytate prepared from oatmeal.

Note: (1) Severe rickets produced by oatmeal (c and h) as compared with that produced by white flour (a and f, b and g).
(2) Severe rickets produced by sodium phytate (d and i, e and j) as compared with condition of control puppies (a and f, b and g)

The first point that emerges from these facts is that the widely accepted view, that the rachitogenic effect of cereals is due to the non-availability of their phytin phosphorus is not true under the experimental conditions described; if it were, phytin and sodium phytate should, in equivalent amounts, produce similar rachitogenic effects, whereas in

the above experiments the former is, if anything, slightly antirachitic while the latter produces the disease. (The slight antirachitic action of phytin has been referred to in earlier publications (Mellanby, 1937; Palmer and Mottram, 1939).) The conclusions of other workers, that the non-availability of phytin P leads to the production of rickets, have been based upon experiments using the high Ca-low P diet normally employed in studying rickets in rats, and the statement is no doubt true under these conditions. As stated above (page 226), such conditions bear little relationship, however, to the diets used in the experiments with dogs or to the normal diet of man.

Starkenstein (1914) put forward the view that the toxicity of phytic acid is due to its conversion of Ca in the body into an un-ionized form. Bruce and Callow (1934) drew attention to the insolubility of calcium phytate and suggested that it was possible to assume that, in low Ca diets, phytic acid might exert an anticalcifying action, not on account of the unavailability of the P but because of its precipitating action on Ca, which was thus reduced to a deficiency level. While, as pointed out, the foregoing experiments do not support the view that the non-availability of phytic acid P has anything to do with the anticalcifying action of cereals under physiological conditions, they do strongly suggest that this anticalcifying effect is due to the action of phytic acid in rendering Ca unavailable. Indeed, most of the facts established by these experiments appear to be explained by this theory. It seems probable that the phytic acid in a rachitogenic cereal like oatmeal immobilizes all, or almost all, of the Ca contained in the cereal by converting it into an insoluble, relatively unavailable, Ca phytate and further, that the excess of phytic acid (over and above that required to precipitate the Ca of the cereal) can exert an additional anticalcifying effect by precipitating other Ca present in the non-cereal part of the diet.

At first sight it might be argued, on the generally accepted view that the phytic acid of cereals is present as the CaMg salt, phytin, that the experiments with sodium phytate do not bear any relation to the effect of feeding cereals; for since, as shown in the experiments, phytin itself is not rickets-producing, it is clear that the rachitogenic action of cereals cannot be ascribed to their phytic acid content, if all that phytic acid is present as phytin. It can be shown, however, both by calculation and by experiment, that such is not actually the case, at any rate as regards oatmeal.

To obtain evidence on this point, a number of samples of the oatmeal used in the feeding experiments were extracted with HCl (10 g. oatmeal, defatted to facilitate extraction, 200 ml. N/2 HCl) by shaking for 2 hours, as in the method of McCance and Widdowson (1935) for phytin estimation. The extract was centrifuged and filtered and an aliquot part was then carefully neutralized to pH 7.0 (in a few cases pH 6.5 or 8.0) by stirring in concentrated NaOH drop by drop. After standing for 2 hours to allow maximum precipitation, the precipitate was centrifuged down, and the amount of Ca and phytate P in the unwashed precipitate and the phytate P in the supernatant fluid were determined.

(The Ca was determined by wet ashing of an aliquot part of the precipitate dissolved in dilute HCl, followed by precipitation and titration as oxalate. The Mg was determined by precipitation as $MgNH_4PO_4, 6H_2O$, followed by gravimetric or colorimetric estimation. The phytic P was determined by precipitation as ferric phytate by McCance and Widdowson's method).

The figures obtained were somewhat variable, but it was found (a) that nearly all the calcium in the defatted oatmeal was extracted by the HCl, values of 60–70 mg. Ca per 100 g. oatmeal being obtained; (b) that the greater part of this Ca came down in the precipitate obtained by neutralizing the extract; (c) that the supernatant liquid still contained a considerable proportion of the phytate of

the above experiments the former is, if anything, slightly antirachitic while the latter produces the disease. (The slight antirachitic action of phytin has been referred to in earlier publications (Mellanby, 1937; Palmer and Mottram, 1939).) The conclusions of other workers, that the non-availability of phytin P leads to the production of rickets, have been based upon experiments using the high Ca-low P diet normally employed in studying rickets in rats, and the statement is no doubt true under these conditions. As stated above (page 226), such conditions bear little relationship, however, to the diets used in the experiments with dogs or to the normal diet of man.

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the oatmeal, and it could be demonstrated that it was capable of precipitating further quantities of added Ca. The average values for phytate P from one sample of oatmeal were 177 mg. per 100 g. oatmeal in the precipitate and 127 mg. per 100 g. in the supernatant fluid. At pH 8.0 similar values were obtained, while at pH 6.5 precipitation was less complete. The results showed that the ratio of Ca to phytate P in the precipitate (usually about 1:3) was considerably smaller than was the case in commercial phytin. The usual figures for commercial phytin are Ca 12%, Mg 1.5%, P 22%. Much of the phytate precipitated from the oatmeal extract was found to be combined with Mg, and analysis of one sample of unwashed precipitate gave the values Ca 56 mg. and Mg. 85 mg. per 100 g. oatmeal. In another experiment in which the pH 7.0 precipitate and supernatant solution were analysed more completely, the following approximate figures were obtained:

	Mg. per 100 g. defatted oatmeal	
	Precipitate	Supernatant solution
Phytic P	122	187
Total P	135	—
Inorganic P	negligible	—
Ca	51	20
Mg	52	103

In this case, the relative amount of phytate, some 60%, remaining in the solution was larger than usual. The conditions in these experiments might perhaps be taken to represent very crudely the processes taking place in the natural digestion of oatmeal, namely, extraction by HCl (more dilute under natural conditions, but assisted by pepsin), followed by neutralization in the intestine. These

experiments suggest that, under such conditions, much of the phytic acid of the oatmeal would be precipitated as phytin, but that this would contain a considerably smaller proportion of Ca than ordinary phytin. Unlike commercial phytin, such a compound might exert some rachitogenic action by exchanging some of its Mg for Ca from the rest of the diet. The number of possible salts of inositolhexaphosphoric acid with more than one metal is very large, and the particular salt which is precipitated from a solution containing the metals will no doubt be determined partly by factors such as pH and the relative concentrations of the bases in the solution. For example, Boutwell (1917) describes the isolation from wheat bran of a phytin with 3.65% Ca and 10.81% Mg, compared with 12% Ca and 1.5% Mg for ordinary commercial phytin. In the experiments in which the Ca-rich commercial phytin is used, an interchange of metals in the opposite direction may possibly occur to some extent, some of the Ca being set free and replaced by Mg. or other bases. This liberated Ca then, perhaps, brings about a slight antirachitic action such as appears to be exerted by commercial phytin.

In addition to this phytate which is precipitated on neutralizing an acid extract of oatmeal, however, there is some 40% or more of the oatmeal phytate still present in solution and capable of precipitating Ca from other sources. It is true that the supernatant liquid still contains a small amount of Ca in solution, but it may well be in an unionized, non-absorbable form. By adding a few drops of $CaCl_2$ and carefully adjusting to pH 7.0, it is easy to show that the solution is capable of precipitating further amounts of Ca.

Apart altogether from the above experiment, it could have been predicted from the chemical analysis of oatmeal that the phytate is not all present as ordinary phytin. Oatmeal contains about 63 mg. Ca per 100 g., and the average

phytate P of the undefatted oatmeal samples was 253 mg. per 100 g. On the basis of the composition of ordinary phytin given above, more than half the phytate would be present in combination with bases other than calcium probably other metals such as sodium, potassium or magnesium, or possibly with protein (see Lindenbaum, 1926; Mnich, 1931). The observation that ordinary phytin is *not* rachitogenic is not then inconsistent with the view put forward that the rickets-producing action of cereals is due to the phytic acid which they contain. This phytic acid may under natural conditions be reasonably expected to interfere with Ca absorption either by actual precipitation of Ca or by otherwise reducing its ionization and diffusibility. The Ca affected may come partly from the cereal *itself*—as it were, a passive rachitogenic effect of the cereal—and partly from *other foods*—an active cereal rachitogenic effect.

This view that the action of cereals is due to interference with Ca absorption accords with the observation that the effect of additional phytate can be prevented by feeding extra Ca, in other words by saturating the phytate and rendering it inactive. It has been abundantly proved that the addition to the diet of a Ca salt such as calcium carbonate or phosphate reduces the rickets-producing effect of oatmeal (Mellanby, 1925; M. Mellanby, 1929). Similar results were obtained by Palmer and Mottram (1937; 1939) using calcium lactate, and the absence of rachitogenic effect in the experiments with phytin itself described above again confirms the fact that Ca counteracts the rachitogenic action of phytic acid.

Incidentally, this theory no doubt explains the observation of Lowe and Steenbock (1936 a) that the addition of calcium carbonate to the diet diminishes the hydrolysis of phytate in the intestine of the rat. The extra Ca in the diet would result in the precipitation of the *whole* of the phytic acid as the Ca salt, and it would be expected that this in-

soluble salt would be hydrolysed in the intestine (phytase) (see page 270) much less readily than the soluble phytates.

Further, the fact that some foods containing phytin show no rickets-producing action, is understandable, for the anticalcifying action of a foodstuff will depend not merely on how much phytate phosphorus it contains but also on how little calcium is present.

Finally, the question must be considered as to what evidence there is that the rachitogenic activity of oatmeal, which has been shown to be retained in the phytic acid fraction prepared from oatmeal, is actually due to the phytic acid itself and not to some impurity in the preparations, for it cannot be claimed that these preparations as fed to the dogs were completely pure sodium phytate or phytic acid, even though the analysis and properties of the crystalline alkaline sodium salt, which were prepared from the solutions used in the feeding experiments agreed closely with those of pure sodium phytate. The evidence that the phytate is the active factor seems strong, however, for the following reasons: (a) the sodium phytate fraction from oatmeal produces a rachitogenic effect of a similar order to that produced by *an equivalent amount of oatmeal itself;* (b) no loss of activity of the phytate fraction is apparent after further purification; (c) sodium phytate prepared from commercial phytin produces a similar effect; (d) the rachitogenic effect of the phytate fraction is antagonized by feeding extra Ca which metal is known to precipitate phytic acid; similarly, the activity of oatmeal itself is counteracted by Ca; (e) treatment of cereal by methods which are known to destroy phytic acid, such as boiling with acid or digestion by phytase (e g. in the autolysis of germinated cereals), lead to a reduction of rachitogenic activity, whereas the activity is not impaired by treatments such as boiling with alkali, which do not break down phytic acid. It should be mentioned, too, that Anderson (1914)

was able to isolate practically pure salts of phytic acid from oats, though the purification involved many stages and the resulting yield was small.

The experiments do not completely exclude the possibility that there may be other rachitogenic factors in cereals, probably other inositol phosphoric esters for example, but there appears to be no clear evidence for this at present.

The question as to whether phytic acid prevents the absorption of Ca by actually precipitating it as calcium phytate, or whether it acts by lowering the amount of ionized or diffusible Ca, cannot at present be answered. It has been shown that most of the Ca of a dilute HCl extract of oatmeal is precipitated by the phytic acid at neutrality. It is not possible to say whether such actual precipitation occurs under the conditions present in the gut, but it seems not unlikely.

From the point of view of practical human nutrition, the experiments described above show clearly that the rickets-producing action of cereals is to be reduced not by increasing the inorganic P of the diet (as would be the case if the cause were lack of available phosphorus), but by increasing the Ca intake, by drinking more milk for example. It appears from these experiments that the rachitogenic action of cereals is only likely to become serious in diets which are on, or below, the borderline of minimum requirements of Ca and vitamin D. It is unfortunate that, for economic reasons, these borderline diets are the ones which so often contain a disproportionately high amount of cereal.

Reviewing the above early work on phytic acid in relation to calcification of bone in young dogs, the situation at that time, i.e. in 1939, may be said to have been as follows:

(a) Phytic acid (inositolhexaphosphoric acid) and neutral sodium phytate prepared from commercial phytin—a calcium magnesium salt of phytic acid—exert a definite

rickets-producing action when added to a non-rachitogenic or slightly rachitogenic diet;

(b) The degree of rachitogenic activity shown by these compounds is roughly comparable with that in oatmeal when fed in an amount equivalent as regards phytic acid P;

(c) The phytic acid P fraction extracted from oatmeal itself shows the same rachitogenic action, and purified neutral sodium phytate prepared from this fraction is equally potent;

(d) The rachitogenic action of sodium phytate, as of cereals, is antagonized by adding extra Ca to the diet, and commercial phytin is slightly antirachitic.

Evidence is given suggesting that the rachitogenic action of cereals is normally due not, as has often been suggested, to the unavailability of their P, but to the action of the cereal phytic acid in inhibiting the absorption of Ca from the alimentary canal.

The amount of phytic acid in oatmeal is approximately twice that required to precipitate the Ca of the cereal at neutrality, and it is suggested that the phytic acid exerts its rachitogenic action by preventing absorption both of the Ca of the cereal itself and of further amounts of Ca from the rest of the diet.

Chapter XIII

SOME POINTS IN THE CHEMISTRY AND BIOCHEMISTRY OF PHYTIC ACID AND PHYTASE

1. Historical Survey

In the last chapter evidence was given to show that a substance in oatmeal having a specific anti-calcifying action on bones is phytic acid. Before continuing the account of further work on the physiological action of this substance, it is necessary to refer to some facts concerning its chemistry and biochemistry in order to give a background to the various procedures that were sometimes adopted in the later experimental work and also for the understanding of its action in the body. An account of this kind may also be useful because so little has been written about phytic acid for the general biologist and medical scientist, many of the facts being still confined to the original papers. In the first place, a bare outline of the early history of the subject will be given. This is taken largely from the excellent review given by Rose in 1912.

Phytic acid was for long a will-o'-the-wisp of botanical chemistry. Knowledge of this substance had its beginning in the discovery by Hartig (1855, 1856), in the seeds of various plants, of small particles which were not starch grains. In 1872 one type of these particles received the special designation by Pfeffer of "Globoid." Pfeffer's colleague, Brandon, found these globoid particles to be free from nitrogen but to contain calcium, magnesium and phosphorus. Organic matter was noted in the particles and the suggestion was then made that the substance was a phosphate combined with a carbohydrate. In 1894 Palladin,

while studying the proteins of sinapis niger, obtained from the fat-free, finely ground seeds a substance which was soluble in 10% sodium chloride, but precipitated on heating. This substance he found to be soluble in cold and insoluble in hot water, and ultimately he obtained a fairly pure product which was rich in phosphorus and contained also calcium and magnesium, but no nitrogen. Palladin's work was confirmed by Schulze and Winterstein (1896) who also expressed the view that the compound under consideration was identical with Pfeffer's globoid particles. In a more detailed paper Winterstein (1897) suggested that the real nature of the substance was inosite-phosphoric acid, since it yielded inosite and phosphoric acid on hydrolysis.

The most extended study of this substance was made by Posternak. He was the first successfully to prepare it in pure form and made a large study of its physical and chemical properties, speculating on its constitution and biological function. Posternak, who did not think in the early stages of this work that inositol (inosite) was present in the molecule, gave the name "phytin" to the substance, and under this trade name it has long been marketed by a Swiss firm.

The suggestion of the actual presence of inositol in the original substance was supported by Neuberg's discovery in 1908 that inositol and furfurol were obtained on mixing "phytin" with phosphoric acid and distilling under reduced pressure, and further, that furfurol could be obtained from inosite. Previous to this work some people thought that inositol itself was synthesised from the products of the hydrolysis of the phytic acid, when it was heated under pressure with mineral acids.

Strong support of the view that inositol was present in phytin was also obtained in 1907 by Suzuki, Yoshimura and Takaishi, who found an enzyme phytase in rice bran capable of breaking phytin down to inositol and phosphoric acid.

This was the first discovery of an enzyme having a phytase action. A further important outcome of the work of Suzuki and his collegaues at this time was the suggestion that phytin was really inositol hexaphosphoric acid. Other suggestions had been made as regards the formula of this substance. For instance, Starkenstein (1914) thought that phytic acid was a complex pyrophosphoric acid compound of inositol, but in 1921 Posternak settled the matter by more detailed investigation. Since that time, it has been accepted that phytic acid is inositol hexaphosphoric acid.

One or two other points in connection with this early work may be mentioned. An important contributor to knowledge of this subject was Vorbrodt (1910), who found that phytase had an extensive distribution in plants, for instance, in grain such as wheat, rye and barley and in larger seeds such as vetch and lentils. He also discovered that phosphorus compounds, especially phytic acid, were particularly abundant in the germ of grains. In view of work to be described later, it is interesting also to know that he found no evidence of the presence of phytase in corn until it had been allowed to germinate, when the enzyme appeared.

Another discovery of interest, which, however, at the time seemed to have but little significance in relation to the work under review, was that of Windisch and Vogelsang who in 1906–7 showed that inositol-phosphoric acid of barley did not pass into beer but disappeared in the malting process. It may be remembered that, early in the physiological experiments concerning the anticalcifying action of cereals (page 222), it was shown that when they were malted, this action was reduced. In the light of more recent experiments, it is easier to see the relationship between Windisch's observation and the observation that the rdchitogenic factor in cereals is reduced by malting (page 293).

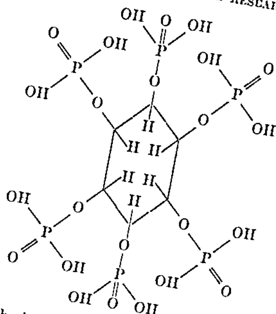
Since phytic acid is a twelve basic acid, it is obvious that

it is capable of forming many salts. Some of these, namely its calcium salts, are of special interest to the present work and therefore a more detailed account of them is given below. The production and chemistry of a number of other salts have been described by R. J. Anderson of the New York Agricultural Experimental Station (1915), who also described inositol phosphoric esters with fewer than 6 phosphoric acid groups in the molecule. Some of these, besides the monophosphate of inositol, may be of physiological interest. Indeed, phytic acid has been of great interest in that laboratory from the time that Hart and Andrews in 1903 provided a method of determining inorganic phosphorus in the presence of phytin and other organic forms of phosphoric acid. Patten and Hart (1904) isolated from wheat bran a magnesium calcium potassium compound and showed that practically all of the soluble phosphorus of such bran is of an organic nature.

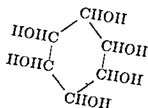
But little is known about the function of phytic acid in plant life, in spite of its abundance in the propagating and growing parts of plants. It is undoubted, however, that plants contain organic phosphorus compounds in other forms. In a general way it can be said that phytin is an insoluble, inert compound, which is especially abundant in plant seeds and is a rich source of phosphorus which can be used in the early stages of growth as needed. The close association of a phytase with the phytin in particular parts of the grain, such as the germ, ensures a mechanism whereby this organic phosphorus compound can be made easily available in a soluble inorganic form, together with the carbohydrate inositol, as the growing seed needs it.

2. Phytic Acid and Its Calcium Salts

Phytic acid is the hexaphosphoric acid ester of inositol (Posternak, 1921). $C_6H_6(OPO(OH)_2)_6$,



It is a 12 basic acid. Phytic acid can be hydrolysed and broken down into its constituent parts, inositol and phosphoric acid, either by boiling with a mineral acid (HCl) or by an enzyme (phytase). The conditions for and the significance of this hydrolytic change will be more closely considered later. Inositol is a monosaccharide and its formula is



It is a substance of undoubted significance in, and is probably essential to, the animal economy, being specially

associated with nerve and muscle tissues, but its function is not known.

The biochemical significance of phytic acid in animal nutrition is undoubtedly closely bound up with its salts, and especially its calcium salts. It is said that phytic acid in plant tissues is usually present as the Ca Mg salt and it is this substance which is commonly referred to as phytin. There is evidence, however, as shown in chapter XII, that there are other compounds of phytic acid in cereals, and certainly in oatmeal, which are not fully neutralized by calcium and rendered insoluble and inert.

In view of the great biological significance of the calcium salts of phytic acid, the following facts, many of which have been described by Hoff-Jørgensen (1944), may be of interest. Hoff-Jørgensen's aim in making a study of this subject was to determine the composition of the calcium salts of phytic acid, when precipitated under conditions similar to those found in the intestine, and secondly to determine the solubility of the said salts. Although this investigation was of high merit and undoubtedly led to an accurate account of the calcium salts of phytic acid produced under the described experimental conditions, a slight warning may well be given, about the extension of *in vitro* results, directly and without question, to apparently similar reactions that may take place in the alimentary canal. The fact is that neither biochemists nor physiologists have the necessary knowledge of all the intestinal factors which may influence even a simple reaction like that resulting in the combination of calcium and phytic acid. It may be, therefore, that Hoff-Jørgensen's results cannot be so directly and wholly applied to *in vivo* conditions. On the other hand, they may, and present results strongly support the view that they do, throw a considerable light on the various changes which phytic acid appears to undergo in the gut.

It is undoubted that the particular calcium salt of phytic acid that is formed depends on the pH of the reacting solution. Thus, Hoff-Jørgensen found that, in solutions of phytic acid and calcium ions in the pH interval of 4.6 to 6.9, amorphous pentacalcium phytate ($C_6H_8O_{24}P_6Ca_5$) was precipitated. He thought, therefore, that this was the form which was found in the gut. Outside the pH range 4.6 to 6.9 the composition of the calcium phytate changed, but he failed to obtain other single calcium salts with a simple Ca:P ratio.

Hay (1942) had previously investigated this problem and he also obtained variations in the calcium salts of phytic acid according to the pH of the reacting fluids. At a pH of 2.5, in the presence of dilute HCl, he obtained the tricalcium salt (Ca_3P_6): at pH 1.2 (in the presence of 50 per cent acetic acid) the tetracalcium salt (Ca_4P_6); at pH 3 to 4.5 either the pentacalcium salt (Ca_5P_6) or a mixture of Ca_4P_6 and Ca_6P_6; at pH 5.8 to the alkaline side of neutrality pH 10, the hexacalcium salt (Ca_6P_6). These results are not in strict accord with those of Hoff-Jørgensen and are much more sharply cut and distinct. For instance, Hoff-Jørgensen thinks the gut phytate would probably be the pentacalcium salt, while Hay thinks it likely to be the hexacalcium salt. The experience in this laboratory with these compounds, although small, suggests that the hexacalcium salt is only formed at a pH of 7.8 upwards.

In view of the experimental results on animals to be described later, it is interesting to note the differences in the manner of precipitation of calcium phytate and calcium phosphate which, according to Hoff-Jørgensen, occur at the pH range to be expected in the alimentary canal. For instance, he finds that the precipitation of pentacalcium phytate is rapid, while the corresponding compound, secondary calcium phosphate ($CaHPO_4$, $2H_2O$), which would probably be formed if phosphoric acid and calcium

ions met at the gut pH, is precipitated only slowly. He also finds there are great differences in the solubilities of pentacalcium phytate, hydroxyl apatite ($Ca_5(PO_4)_3OH$), and secondary calcium phosphate in 0.2 M. NaCl at pH intervals 4.4 to 7.6. Thus, both hydroxyl apatite and calcium phytate are practically insoluble at the neutral pH but, of the two, the former is the less soluble on the acid side of neutrality. The solubility of pentacalcium phytate decreases rapidly as the temperature rises. Relatively to these substances, secondary calcium phosphate is much more soluble. On the basis of these physico-chemical considerations Hoff-Jørgensen concludes that, when the stomach contents containing Ca and phosphate are emptied into the intestine; a precipitation of secondary calcium phosphate forms slowly, some remaining in solution. On the other hand, calcium phytate formed in the gut under the same conditions is precipitated quickly, is only slightly soluble, is not liable to form supersaturated solutions, and thus greatly reduces the available calcium ions in the intestine. From these facts it would appear likely that phytic acid is more powerful in completely immobilising calcium in the alimentary canal than phosphoric acid, a property which is supported by the biological results which will be described later. It is probable that, when phytate is present in the gut as an insoluble calcium salt, it is only hydrolysed with great difficulty by phytase and is not easily available. On the other hand, when sodium phytate is the only source of phosphorus available to an animal, there is evidence from the rat experiments of Krieger, Bunkfeldt and Steenbock (1940 a) that it can be dealt with by the digestive mechanism. The same workers (1940 b) also showed that, when Ca phytate is the source of Ca, rats are able to utilize the Ca of this insoluble compound. It appears therefore possible that under suitable conditions these animals might be able to utilize both the Ca and the P of insoluble Ca phytate. (See also p. 329.)

3. The Hydrolysis of Phytate to Inorganic Phosphate and Inositol

The hydrolysis of phytate to two compounds, inositol and phosphate, which are essential for the well-being of the animal, is a change of important biochemical significance. In the experiments to be described later detailed information on the conditions affecting this hydrolytic change and its rate was needed and therefore this subject will now be discussed.

Phytate can be hydrolysed in two ways, (1) by a mineral acid such as HCl, and (2) by the enzyme phytase. In the case of phytase, at least three sources are known, cereals, yeast and the alimentary canal. Although reference will be made to all three types of enzymic hydrolysis, special attention will be given to the destruction of phytate in the whole grain, in the grain after grinding, during germination and after autolysis. The phytases of cereals and of yeast are similar in that both are most active at a pH of 4.5. The phytase detected in the intestinal mucous membrane of some animals, however, has its optimum activity at pH 7.6 and is clearly different from the others described (Fig. 49).

(a) *Hydrolysis by HCl*

When sodium phytate is boiled with 1% HCl it is slowly hydrolysed and phosphate is liberated, as is shown in Fig. 44. After 18 hours' boiling, about 95 per cent of the phytate is hydrolysed. The slow rate of hydrolysis of phytate when oatmeal is boiled with the same dilution of HCl is also shown, and it is seen that, even after 18 hours' boiling there are left unhydrolysed 65 mg., i.e. about 23 per cent of the 280 mg. of phytate phosphorus in the original oatmeal; so that the hydrolysis of phytate, whether as sodium phytate or as the compound in oatmeal, seems to be similarly affected by mineral acids.

(b) *Hydrolysis by enzyme (phytase)*

(i) *Phytase of cereals.* There have been many reports of the presence of a phytase in cereals since the original account by Suzuki, Yoshimura and Takaishi (1907) was published. The work most often quoted is that of Adler (1915), who suggested that the optimum conditions for the

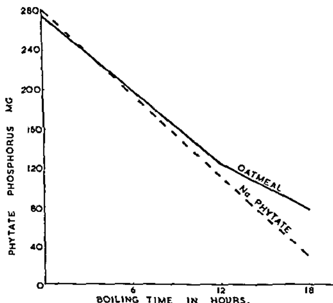


Fig. 44 Hydrolysis of phytate to phosphate by boiling with 1% HCl (a) oatmeal, (b) sodium phytate

action of phytase were pH 5.4 and a temperature of 43–58°C. Since then Pedersen (1940), Schulerud (1944, 1947) and Hoff-Jørgensen (1947) have stated that the optimum pH is 5.2, Møllgaard et al. (1946) have suggested 5.0, and McCance and Widdowson (1944) have demonstrated a pH of 4.5 as the best. Some of the discrepancies may be due to variations in the buffer solutions employed, full details of

3. The Hydrolysis of Phytate to Inorganic Phosphate and Inositol

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Phytate can be hydrolysed in two ways, (1) by a mineral acid such as HCl, and (2) by the enzyme phytase. In the case of phytase, at least three sources are known, cereals, yeast and the alimentary canal. Although reference will be made to all three types of enzymic hydrolysis, special attention will be given to the destruction of phytate in the whole grain, in the grain after grinding, during germination and after autolysis. The phytases of cereals and of yeast are similar in that both are most active at a pH of 4.5. The phytase detected in the intestinal mucous membrane of some animals, however, has its optimum activity at pH 7.6 and is clearly different from the others described (Fig. 49).

(a) *Hydrolysis by HCl*

When sodium phytate is boiled with 1% HCl it is slowly hydrolysed and phosphate is liberated, as is shown in Fig. 44. After 18 hours' boiling, about 95 per cent of the phytate is hydrolysed. The slow rate of hydrolysis of phytate when oatmeal is boiled with the same dilution of HCl is also shown, and it is seen that, even after 18 hours' boiling there are left unhydrolysed 65 mg., i.e. about 23 per cent of the 280 mg. of phytate phosphorus in the original oatmeal; so that the hydrolysis of phytate, whether as sodium phytate or as the compound in oatmeal, seems to be similarly affected by mineral acids.

(b) *Hydrolysis by enzyme (phytase)*

(i) *Phytase of cereals.* There have been many reports of the presence of a phytase in cereals since the original account by Suzuki, Yoshimura and Takaishi (1907) was published. The work most often quoted is that of Adler (1915), who suggested that the optimum conditions for the

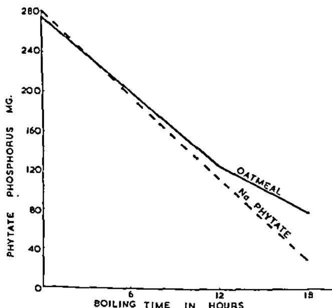


FIG. 44. Hydrolysis of phytate to phosphate by boiling with 1% HCl (a) oatmeal, (b) sodium phytate

action of phytase were pH 5.4 and a temperature of 43–
[illegible] Pedersen (1940), Schulerud (1944, 1947)
(1947) have stated that the optimum
al. (1946) have suggested 5.0, and
(1944) have demonstrated a pH
[illegible] discrepancies may be due to
[illegible]ons employed, full details of

3. THE HYDROLYSIS OF PHYTATE TO INORGANIC PHOSPHATE AND INOSITOL

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and then to grind the grain several times rather than attempt to reduce it to a fine powder in one process. Figures are given (Table 8) to show the rate of hydrolysis of phytate by the phytase of wheat under these conditions, and also of the whole grain before grinding.

It will be seen how relatively slow is the hydrolysis of phytate in the whole intact grain, there being still about 17%, or 38 mg. of phytate P, at the end of 12 hours. In the ground grain, on the other hand, all the phytate is hydrolysed within 1 hour. The sum of the phosphorus of phytate and inorganic phosphate at all times during the hydrolysis is not constant, there being discrepancies both in the ground grain and in the whole grain experiment. These may be due to defective extraction of the grain, especially the hard whole grain after only 1 or 2 hours' standing. It is possible, however, especially in the ground grain experiment, that the difference is due to the fact that there is an intermediate stage of breakdown of phytate, such as a phosphate of fewer than 6 molecules attached to inositol, when the P is not estimated either as phytate or as inorganic phosphate. Thus, its partial breakdown may proceed more quickly than its complete conversion to inorganic phosphate. A graph giving the rates of hydrolysis of phytate in wheat by phytase under these two conditions is shown in Fig. 45. All cereals do not have such a high phytase content as wheat under these conditions, and the relative activities vary considerably, as can be seen by reference to Table 9.

In all the tests reported below the conditions were the same: 5 g. of grain were suspended in 25 ml. of acetate-acetic buffer and shaken for the whole of the incubation period (Temperature 45°C.; Buffer pH 4.5). All grains, with the exception of corn, were ground to approximately the same degree of fineness. Sample 2 of corn was somewhat coarser as it proved difficult to grind finely without

which have not always been included in the literature. It has been found in the present work that, in order to demonstrate the action of a cereal phytase, such as that of wheat, under optimum conditions, the grain should be

TABLE 8

*Rate of hydrolysis of wheat phytate by wheat phytase (suspended in water at 45°C. and pH 4.5)**

Time of incubation	Mg. of phytate phosphorus per 100 g. wheat	Mg. of inorganic P per 100 g. wheat
	Ground	
(minutes)		
0	232	30
7	190	40
15	137	70
30	98	125
45	50	175
60	0	215
120	0	253
	Whole	
(hours)		
0	232	30
1	121	85
2	99	85
4	91	140
6	81	150
12	38	225

* A pH of 4.5 was maintained by using a sodium acetate-acetic acid buffer (Walpole, 1914).

ground and suspended in water at a temperature of 45°C. and pH 4.5. Phytase of cereals in the presence of water is heat-labile and care must be taken to see that undue heat is not produced during the grinding of the grain. In this laboratory it has been usual to cool the grinding machine

producing considerable heat. Sample 1 was purchased as maize meal and was commercially ground.

Table 9 shows that under the conditions employed in the tests, (1) rye and wheat had the greatest phytase activity; in rye, the whole of the phytate P (168 mg. per 100 g.) was hydrolysed in 45 minutes, and in wheat, the corresponding time for the hydrolysis of 232 mg. phytic acid phosphorus per 100 g. wheat was 60 minutes. (The high phytase content of rye and wheat had previously been described by Pedersen (1940).) (2) The phytase of oats and of yellow and white corn acted much more slowly, so that even after 12 hours a large part of the phytate remained intact in these grains.

It is of interest to note that the two most powerful rachitogenic cereals—oats and corn—have the least phytase action under these conditions, whereas wheat and rye, with a high phytase activity, have not such a powerful rickets-producing effect. It is, however, difficult to compare the rachitogenic effect of rye with that of other cereals, for it has long been known that rye germ may contain a form of active vitamin D (Mellanby, Surie and Harrison, 1929).

Differences similar to those between whole and ground wheat, as regards rate of destruction of phytate, were found in all the grains tested (see Table 8). It was thought possible that the variations might be due to the mechanical effect of the grinding in that it broke up the grain, thereby allowing the phytate and the phytase to come into contact. Fig. 46 shows that this was not so in the case of oats, for ground grain stood in a moist atmosphere was less effective in hydrolysing the phytate than either the ground or the whole grain in water.

Reference was made in Chapter XI to the fact that the process of malting reduced the rickets-producing effect of cereals, so that, when it became probable that the substance responsible for this anticalcifying action of cereals was

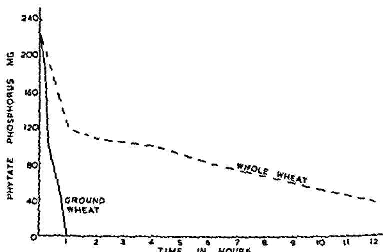


FIG 45. Rate of hydrolysis of wheat phytate by wheat phytase. (The wheat was stirred in water at a pH of 4.5 and a temperature of 45° C.)

Note: The relatively slow hydrolysis of the phytate in the intact grain as compared with that in the ground grain.

TABLE 9

The rate of hydrolysis of cereal phytate by the phytase in various ground cereals at 45°C. and pH 4.5

Time of incubation		Mg of phytic acid phosphorus per 100 g.						
Hours	Minutes	Wheat	Rye	Barley	Oats	Yellow corn 1	Yellow corn 2	White corn
0	0	232	168	198	194	238	212	180
0	7	190	156	192	—	231	—	—
0	15	137	125	164	—	225	—	—
0	30	98	23	110	—	222	—	—
0	45	50	0	75	—	219	—	—
1	0	0	0	36	181	219	194	175
2	0	—	—	0	156	206	189	153
4	0	—	—	—	154	—	180	141
6	0	—	—	—	152	—	176	—
12	0	—	—	—	144	—	157	130

poses of growth. This hydrolytic change is one of the important chemical facts associated with the early development and growth of the plant. In the present work, however, the process was studied from the point of view of the nutritional effect of the end-products of hydrolysis, and not from its more important aspect in plant life. Of the changes to be described, those occurring during steeping and germination can probably be regarded as physiological, whilst those taking place after the grinding of the germinated grain are due to various factors, partly no doubt to enzyme in the grain and partly to moulds.

Table 10 shows the effect of germination on the phytic acid concentration in various grains. All the grains were steeped for 2 days at room temperature and then germinated at 25° C; after 7 days' germination, they were ground and allowed to stand at 25°C. in a moist atmosphere for a further 4 days, during which period the mass of cereal often showed heavy bacterial and yeast growths. In the case of rye and corn there was particularly large growth at this stage and it was difficult to say to what extent the changes in the phytate of the corn were due to the activity of microörganisms and to the cereal phytase respectively. Except in the case of rye, complete hydrolysis of phytate did not take place during 7 days' germination, but when the grain was ground and allowed to autolyse, phytate disappeared quickly from all the grains tested except oats. Even during germination the above mentioned sample of rye developed a large growth of mould. This may not have been of any significance, but it must be noted that the degree of mould growth varied greatly in the different cereals and that those with the greatest destruction of phytate were those on which the greatest mould growth was found.

Although, as seen in Table 8, in wheat the phytase shows an intense activity when the grain is ground and suspended

phytate, it was a matter of great interest to know exactly how phytate in cereals was affected by different stages of the

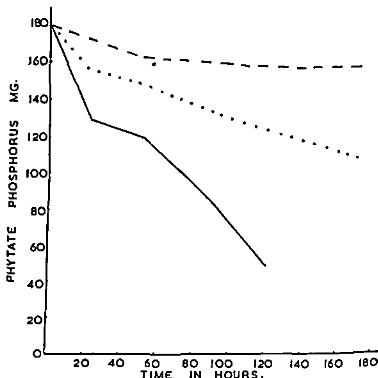


FIG 46. The rate of hydrolysis of oats phytate by oats phytase
——— Ground oats in water; temperature 37°C.
Whole oats in water; temperature 37°C.
.... Ground oats in a moist atmosphere; temperature 37°C.

Note 1. The relatively slow hydrolysis of phytate in oats even when ground. (c. f Fig. 45)

2 The slow hydrolysis of phytate in oats compared with that in wheat (Fig. 45) indicating a much lower phytase content in oats than in wheat (see p 260).

malting process. It is undoubted that the gradual and slow hydrolysis of phytate during germination allows the phosphorus to become available in the inorganic form for pur-

ature. After 7, 9 and 11 days samples of germinating oats were finely ground and allowed to autolyse for 1, 2, 3 and 4 days. Thus it was possible to trace the change from phytate to inorganic phosphate and to determine the increasing solubility of phosphorus and calcium compounds. The results are given in Fig. 47.

It is of interest to note (Fig. 47a) that, even after germinating for 9 or 11 days, the developing oats still contain much phytate, i.e. about 100 mg. phytate P per 100 g. of grain, as compared with 185 mg. per 100 g. of oats before steeping, but when oats are then ground and allowed to autolyse, the total destruction of the phytate occurs within 1 or 2 days. If the germination period is reduced to 7 days, the autolytic breakdown of phytate to phosphate is much slower, and even after a further 4 days of autolysis there is still a little phytate present.

These tests, which show that 7 days' germination followed by 4 days' autolysis destroys less phytate than does 9 days' germination followed by 2 days' autolysis (both having a total treatment of 11 days), indicate that some change, involving either the production or the activation of phytase, takes place during germination. This fact has been previously referred to in the case of corn and other grains (Vorbrodt (1910) and Møllgaard et al., 1946). Fig. 47b shows the increase in the water-soluble phosphorus during germination and autolysis; at the end of the treatment practically all the phosphorus of the grain has appeared in the watery extract. It will also be seen that the phosphorus-containing compounds are not alone in being affected by germination and autolysis. Accompanying these P changes, calcium ions are freed into solution and increase from 20 mg. to about 100 mg. Ca per 100 g. oats (Fig. 47c). Clearly, this point must also be considered in comparing the effect on calcification of diets containing either oats or the products of oats resulting from germination and autolysis.

in water at 45°C. and at pH 4.5, during germination the hydrolysis of phytate is relatively slow, being, in fact, comparable to that of corn.

TABLE 10

Effect of germination on the phytate phosphorus of cereals*

Treatment	Mg. of phytate phosphorus per 100 g					
	Wheat	Rye	Barley	Oats	Yellow corn	White corn
Whole grain untreated.......	232	168	198	195	212	180
1. Grain steeped and germinated, 1 day	200	121	156	160	164	175
2. Grain steeped and germinated, 2 days	200	59	139	158	131	153
3. Grain steeped and germinated, 3 days	161	—	117	149	117	133
4. Grain steeped and germinated, 4 days	153	12	114	157	115	115
5. Grain steeped and germinated, 5 days	139	0	82	130	76	115
6. Grain steeped and germinated, 6 days	125	—	—	134	—	—
7. Grain steeped and germinated, 7 days	110	—	69	131	70	90
8. As 7, then ground and autolysed, 1 day	0	—	6	77	0	0
9. As 7, then ground and autolysed, 2 days	0	—	0	24	0	0
10. As 7, then ground and autolysed, 3 days	0	—	0	20	0	0
11. As 7, then ground and autolysed, 4 days	0	—	0	19	0	0

* Temperature 22 to 26°C.

In the next test to be described, oats were steeped for three days in water at room temperature (16°C. to 20°C.) and were then allowed to germinate at the same temper-

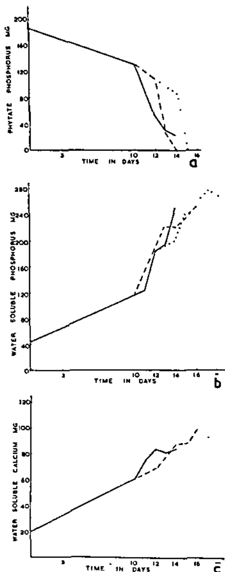

PHYTATE PHOSPHORUS MG
TIME IN DAYS
a
WATER SOLUBLE PHOSPHORUS MG
TIME IN DAYS
b
WATER SOLUBLE CALCIUM MG
TIME IN DAYS
c

FIG. 47

It was noted above that during the germination of grain, especially of rye, and even more so during autolysis of the ground, germinated cereals, moulds grew rapidly. An attempt was therefore made to see whether phytate was hydrolysed by incubating it with yeast, whether in fact yeast contained phytase.

(*ii*) *Phytase of yeast.* Compressed baker's yeast proved to be a rich source of phytase (Mellanby, 1944), and when it was allowed to act on sodium phytate there was a rapid conversion of the phytate to phosphate (Fig. 48). This method is very useful, as will be seen later (Chapters XIV and Appendix II, p. 435), for preparing large quantities of hydrolysed phytate for feeding experiments. It can also be used to hydrolyse the phytate of cereals which have a low phytase content. The following figures show the effect of

---

FIG 47. Effect of germination and autolysis on the phytate, phosphate and calcium of oats.
(a) Hydrolysis of phytate. (b) Increase in water soluble phosphorus. (c) Increase in water soluble calcium.

Treatment of oats

	Steeped	Germinated	Autolysed after being minced
———	3 days	7 days	4 days
- - - - - -	3 days	9 days	4 days
	3 days	11 days	4 days

Note. In (a), that,
1. The rate of phytate hydrolysis is slow during germination but increases when the germinated grain is minced and allowed to autolyse.
2 The phytase increases in amount during germination. (cf. 7 days' germination + 4 days' autolysis with 9 days' germination + 2 days' autolysis.
In (b), that,
1. During germination the increase in water soluble P is about equal to the disappearance of phytate P (cf. (a)).
2. During the autolysis following mincing the increase of P is greater than *the disappearance of phytate.*
In (c), that,
1. The increase in water soluble Ca both during germination and autolysis.

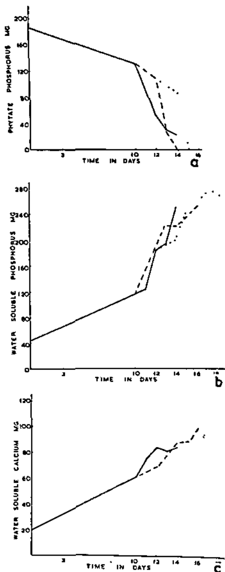

PHYTATE PHOSPHORUS MG
TIME IN DAYS
a
WATER SOLUBLE PHOSPHORUS MG
TIME IN DAYS
b
WATER SOLUBLE CALCIUM MG
TIME IN DAYS
c

FIG. 47

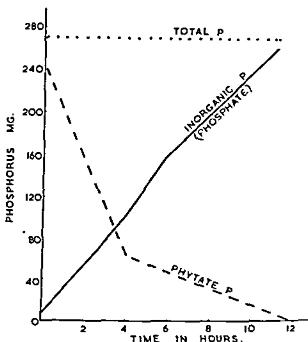


FIG. 48. Hydrolysis of sodium phytate to inorganic phosphate by the phytase of yeast.

Na phytate containing 240 mg. of P was incubated with 5 g. of compressed baker's yeast in 240 ml. of water at pH 4 5 and temperature of 45°C.

incubating oatmeal with and without yeast at pH 4.5 and a temperature of 45°C. for 44 hours.

Treatment of Oatmeal	Phytate phosphorus remaining (mg. per 100 g.)
Untreated	266
Incubated without yeast	213
Incubated with 5% yeast (compressed baker's)	27

There are great differences in the phytase contents of various batches of yeast.

(*iii*) *Phytase in the alimentary canal.* From the point of

view of animal physiology, the question of the amount, distribution and conditions controlling the activity of phytase in the alimentary canal is one of importance and yet little is known about it. Until recently indeed, it was thought that all hydrolysis of phytic acid in the intestines was due to bacterial activity. As will be seen later, however, this is almost certainly not the case (see Chapter XV). Patwardhan (1937) found phytase in the mucous membrane of the alimentary canal of the rat. His observation has been confirmed by Spitzer and Phillips (1945 a), and also in the present work, but no success has been obtained in the search for phytase in the alimentary mucous membrane of the dog, cat, ferret or chicken. Spitzer and Phillips (1945 b) have found phytase in the wall of the rumen of bovines.

The following method is used in this laboratory for making preparations of phytase from the mucous membrane of the alimentary canal. The intestines are quickly removed after death, washed through with distilled water and the mucous membrane scraped off. The scrapings are intimately mixed and stirred with twice as many ml. of acetone as there are g. of scrapings; this is repeated once. They are then extracted once with a mixture of acetone and ether, then twice with dry ether, and the ether allowed to evaporate. The powder is stored in a cool place and retains a good deal of its phytase activity for some months. The effect on the phytase activity of the powder of varying the pH is shown in Fig. 49a. The buffer solutions used in the pH interval 4 to 5.8 are the sodium acetate-acetic acid mixtures of Walpole (1914) and, for the higher pH values, the veronal-HCl mixture of Michaelis (1930). The optimum pH is in the region of 7.6 to 7.8, as was stated by Patwardhan, and this phytase therefore is quite distinct from the cereal phytase previously described. Fig. 50 shows the relative phytase activity of the mucous membrane of the various animals tested.

There have been several reports of the reduced availability of phytic acid P as the dietary Ca is increased (Lowe and Steenbock, 1936 a; Krieger and Steenbock, 1940). As is shown below (Fig. 49a), sodium phytate is easily destroyed by rat intestinal phytase. If, however, Ca is added to the substrate, then no destruction of phytate takes

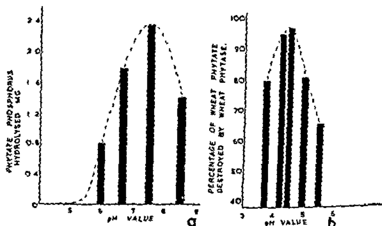


FIG. 49 Effect of varied pH on (a) intestinal (rat) and (b) cereal phytase activity.

Figure (a) represents the *amounts* of phytate P hydrolysed by 30 mg of dried rat mucous membrane in 6 hrs. at 37°C. when the pH of the substrate was varied.

Figure (b) represents the *percentage* of wheat phytate P hydrolysed in 2 hrs. by the phytase contained in the wheat when the pH of the substrate was varied

Note: 1. The optimum pH for rat intestinal phytase is in the region pH 7.6–7.8, and

2. The optimum for the cereal phytase is about 4.5, showing very clearly that the two enzymes are distinct.

place. Calcium phytate is insoluble at a pH of 7.6, and in vitro at least the phytase cannot attack the precipitated phytate. On the other hand, if the pH is lowered so that the calcium phytate is in solution, then it is outside the range at which the rat intestinal phytase is active. It can be seen, therefore, that adding calcium to sodium phytate effec-

tively protects it in vitro from any attack by rat phytase. This 'protection' of phytic acid by calcium against phytase is the probable cause of the reduced availability of phytic

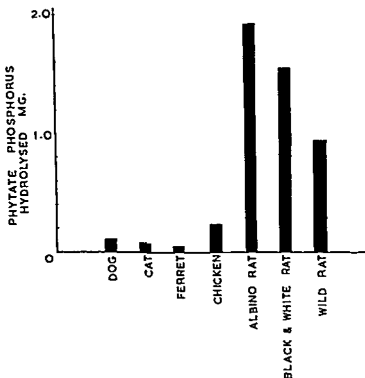


Fig. 50 Hydrolysis of sodium phytate produced by equal weights of dried mucous membrane of alimentary canal of different animals. (All incubated at 37°C. and pH 7.6 for 4 hours.)
Note: Only mucous membrane of rat gut seems to show definite phytase activity at this pH.

acid in rats and possibly in other animals when the dietary calcium is increased. The question of phytase in the gut became later a subject of greater interest and study (p. 380).

4. The Distribution of Phytate and Phytase in Cereals

(a) *Phytate in cereals and cereal products*

Having discussed various methods by which phytate can be hydrolysed, the amounts of this substance in various grains and its distribution within the grain will be con-

TABLE 11

Phytate phosphorus and calcium contents of various grains

	Mg per 100 g. of grain					
	Phytate P				Total Ca	
	Pedersen (1940)	Common (1940)	McCance and Widdowson (1940–1944)	Mellanby	Sherman (1935)	Mellanby
Oats	228	218–312	182–233	180–200		101
Oatmeal		242	266	230–330	69	48–69
Wheat	273	111–305	233–242	233–260	45	48
Corn (yellow)		149–276		212–225		23
Corn (white)				180		29
Corn (unspecified)	199–270				20	
Rye	250		217–242	168	55	65
Barley	182–205	178–217	260	198	43	67
Yellow corn meal		277–314		238		
Rice (polished)			41–60	36		

sidered. The above figures (Table 11) show the average phytate P contents of grains examined in this investigation, together with those published by some other workers.

As can be seen in Table 11, the phytate P of the same type of grain varies. It is possible that much of the variation is due to the maturity of the grain when gathered, for it has been shown by Earlly and de Turk (1944) that phytic acid phosphorus is probably a storage product in grain and that the percentage of phosphorus present as phytin increases as

the grain matures. Its distribution in the different parts of the grain has been studied only on oats and wheat, but it is likely that these results are typical of all grains. The details shown in Tables 12 and 13 were obtained from milled samples of wheat.

TABLE 12

Distribution of phytate in wheat and wheat products

	Mg. phosphorus per 100 g					
	Coarse bran (7.5%)	Fine bran (pollards) (8.0%)	Fine bran, germ and outer endosperm (middlings) (7.5%)	Straight run flour (77%)	Whole grain (100%)	Germ
Phytate phosphorus .	994	828	531	52	234	546
Total phosphorus	1,135	955	825	119	312	
Phytate phosphorus as % of total phosphorus .	88	86.5	64.5	43.6	75	

The figures underneath the headings, bran, flour etc. represent the percentate amount of these products obtained from the grain.

TABLE 13

Amounts in mg of phytate phosphorus per 100 g. of whole wheat in the various layers

Coarse bran	Fine bran (pollards)	Fine bran, germ and outer endosperm (middlings)	Straight run flour	Whole wheat
74	66	40	40	234

The part of the wheat richest in phytate is the bran, and the endosperm is the poorest. In straight run flour the concentration is only about 1/18th that in bran, and in patent flour, which is almost pure endosperm, only 1/52nd. Since, however, the bran and pollards form only about 15

per cent. of the grain and the endosperm 85 per cent, the ratio of the total amounts of phytate in these two parts of the grain, i.e. bran and endosperm, is more like 2 to 1. The phytate is also highly concentrated in the germ but, as this forms only about 2.5 per cent of the grain, its total content of phytate is small.

TABLE 14

Distribution of phytate in oats and oat products

	Mg. phosphorus per 100 g.					
	Husk (30 2%)	Germ (1.80%)	'Endosperm' etc. (68 0%)	Whole oats (100%)	Oatmeal	Oatflour
Phytate phosphorus	0.0	700	250	187	262	160
Total phosphorus	39.5	1,289	360	305	385	204

The figures underneath the headings, husk, germ, etc. represent the percentage amount of these products obtained from the grain.

Here again the richness of the germ in phytate is evident, and its absence from the husk of oats is to be noted. The endosperm etc., referred to above, which represents 68.0 per cent. of the total grain, is not really comparable with the structure so called in the case of wheat, as it contains layers analogous to the bran and aleurone layers of wheat. It is of interest to note that during the war a product known as oatflour was milled in Britain in an attempt to produce from oats something comparable to wheaten flour. The phytate P content was about 160 mg. per 100 g. The product probably consisted largely of the part of the oat described above as endosperm etc., without some of the more branlike layers, and did not contain the husk or germ.

Oatmeal consists of whole oats without the husk and its phytate P content is high, varying between 220 and 300 mg. per 100 g.

(b) *Phytase in cereals and cereal products*

It might be expected that the concentration and distribution of phytase in different parts of the cereal grain would accompany the phytate, since its prime function would presumably be to hydrolyse this substance. This is generally true in the case of wheat, as can be seen from Table 15. Portions of 2 g. of different parts of the wheat grain were suspended in water (after careful grinding) and sodium phytate was added to bring the final concentration up to 20 mg. of P as phytate P. The amount of phytate

TABLE 15

Distribution of phytase in different parts of wheat grain
(All incubated at 45°C. for 2 hours at pH 4.5)

Part of wheat grain	Phytate phosphorus in mg			
	Originally present in grain product	Added as Na phytate	Total in tube	Amount hydrolysed by phytase in grain product
Coarse bran .	19.87	0.13	20.0	17.98
Fine bran (pollards). ..	16.56	3.5	20.06	17.39
Fine bran, germ and outer endosperm (middlings)	10.624	9.558	20.182	17.002
77% flour .	1.031	19.332	20.363	1.860
50% flour .	0 38	19 98	20.36	0.363

remaining after 2 hours' incubation at a temperature of 45°C. and pH of 4.5 was estimated and the amount of phytate hydrolysed by phytase determined.

The figures in Table 15 were obtained from millings of the same grist and, although the total hydrolysis was rather less than was expected from experience with whole wheat, it shows clearly that those parts of the grain containing the highest phytate, i.e. bran, germ, etc., also contain the most phytase.

It may be objected that the above results do not represent a reliable picture owing to the fact that added phytate may be less easily destroyed than the phytate present in the wheat. However, it is to be noted that both pollards and middlings, especially the latter, contain sufficient phytase to hydrolyse not only their own phytate but also some of the added phytate. It seems evident, therefore, that bran and wheat germ are rich in both phytate and phytase and that wheat endosperm is comparatively poor in these substances.

As regards oats, it was shown above that the husk is devoid of phytate and that the germ is very rich in this substance. Tests were made to determine the relative

TABLE 16

Distribution of phytase in different parts of the oat grain

In 24 hours:
1.175 mg. of phytate P were destroyed by 2 g. of husk;
19.600 mg. of phytate P were destroyed by 2 g.* of germ;
2.700 mg. of phytate P were destroyed by 2 g. of 'endosperm' etc.

* Actually only 0.5 g. of germ was used in the test and 4.9 mg. of phytate were destroyed.

phytase contents of the various parts of the grain. The above figures were obtained in one test in which all solutions of phytate were buffered to pH 4.5 and incubated at 45°C.

The very high concentration of phytase in the germ of oats is again obvious, so that it can be confirmed that, generally speaking, high phytase also accompanies high phytate in particular parts of the oat grain. It is of interest, however, that the husk, which forms about 30 per cent of the grain, is devoid of phytate P, yet apparently contains a little phytase. Although it is generally true that the greatest concentrations of phytate and phytase are found

in the same portion of the grain, it does not follow that cereals rich in phytate are also rich in phytase. For instance, oats and corn are rich in phytate P but poor in phytase. Rye, on the other hand, contains rather less phytate P than oats and corn, but its phytase content is very high (see Tables 9 and 10).

5. The Fate of Phytate in Wheat and Oats When Prepared for Human Consumption

It is a matter of interest to see the contrast between the effect on the original phytate content of the grains of processing and cooking wheat and oats for human food (Mellanby, 1944). In the case of wheat, the process of milling and then baking the flour into bread is to be considered; in the case of oats, the process of grinding into oatmeal and the conversion of oatmeal into porridge or oat cake. Oats and wheat contain, in their natural state, about the same amount of phytate P, i.e. about 200 mg. per 100 g. grain.

Now the husk which is removed in the preparation of the oatmeal forms about 30 per cent. by weight of the grain, and, since this husk contains no phytate, the percentage of this substance in the dehusked grain is correspondingly higher, namely up to 270 mg. or more of phytate phosphorus per 100 g. oatmeal. On the other hand, in processing wheat to flour, assuming that the wheat-meal flour is of 85 per cent extraction, the 15 per cent removed in the milling is largely made up of the coarser bran, which is very rich in phytate. Thus wheat-meal flour of 85 per cent extraction would contain about 120 mg. or even less phytate phosphorus per 100 g. flour. Starting, therefore, with oats and wheat grain, both containing 200 mg. phytic acid phosphorus per 100 g. of grain, by the time each preparation reaches the cook there is a great difference between their contents: 270–300 mg. phytate phosphorus in oat-

meal and 120 mg. in national wheat-meal flour; and this is not the end of the story.

In preparing these products for the consumer, the cook again alters their relative phytate content. The oatmeal is boiled as porridge, its small phytase content is destroyed and the phytate remains at the same high figure. In the case of wheaten flour, the matter is different. The high phytase content gets a chance of destroying phytate in the flour during the period when the dough is standing. In this process, however, the flour phytase is not the only phytase present. The added yeast is also rich in this enzyme, and it may, under some conditions, assist the hydrolytic breakdown of phytate. Whether it does so assist seems to depend on the method of bread-making adopted. If the amount of yeast added is relatively large (2.1 per cent of flour) and the time of standing of the dough short (2 hours), the yeast may add largely to the phytate hydrolysis. If, however, the yeast added is small (0.6 per cent of flour) and the dough rising-time long (6 hours), the yeast phytase may be ineffective. The phytase action of both flour and yeast is greatly increased as the pH is lowered towards 4.5, and it is possible that the additive effect of the larger amount of yeast may be partially or wholly explained by this change in pH. The accompanying results obtained on baking bread with flour at two levels of phytate content illustrate this effect of yeast.

It will be seen from Table 17 that, when the amount of yeast added to the flour was relatively large and the rising time short, the yeast phytase, in both specimens of flour, increased the amount of phytate hydrolysed (estimated as P) by 21 mg. P per 100 g. flour. When the yeast concentration was relatively low, however, and the rising time longer, the action of the yeast phytase was not obvious.

The destruction of phytate in flour by phytase during different modes of cooking has been described by Widdow-

son (1941). Some of the results obtained were as shown in Table 18.

It is clear that, in consequence of the phytase present both in flour and yeast much of the phytate in flour can be

TABLE 17

Hydrolysis of phytate in breadmaking by phytase of flour and of yeast

Conditions under which bread is made			Mg. phytate phosphorus per 100 g flour			
Amount and state of yeast	Rising time	pH	In wheat meal flour	In bread	Hydrolysed by phytase	Probably hydrolysed by yeast added
Specimen 1:						
High, live	Short	5.5	75	29	25	21
High, boiled	Short	5.5	75	50	25	0
Low, live	Long	5.5	75	26	46	3
Low, boiled	Long	5.5	75	29	46	0
High, live	Short	4.5*	75	0	75	0
High, boiled	Short	4.5*	75	0	75	0
Specimen 2:						
High, live	Short	5.8	174	77	76	21
High, boiled	Short	5.8	174	98	76	0
Low, live	Long	5.8	174	60	114	0
Low, boiled	Long	5.8	174	57	117	0

High and low yeast = 2 1% and 0.6% respectively.

Short and long rising time = 2 hours and 6 hours respectively.

* pH of 4 5 obtained by addition of HCl.

converted into inorganic phosphate in baking. About 60 per cent. is, on the average, so converted in the bread made from flour of 82½ per cent. extraction used in Britain today.

It will be seen from Table 18, however, that the percent-

age of phytate (85 per cent) broken down in the preparation of bread from flour of 70 per cent extraction is much higher than that broken down in bread made from wheat-

TABLE 18

Destruction of phytic acid phosphorus during cooking of foods

Nature of flour	Nature of cooked product	Amount of phytic acid P originally present in mg. per 100 g flour	Percentage destruction of phytic acid	Actual* destruction of phytic acid P in mg per 100 g flour
White (70% extraction)	Bread made with yeast	51	85	43.4
National wheatmeal (85% extraction)	Bread made with yeast	127	69	87.6
Wheatmeal (92% extraction)	Bread made with yeast	214	31	66.3
Wheatmeal (92% extraction)	Baking powder bread	214	5	10.7
Wheatmeal (92% extraction)	Steamed pudding	214	16	34 2
Wheatmeal (92% extraction)	Pastry	214	0	0
White flour with added sodium phytate	Baking powder bread	214	15	32.1
White flour with added sodium phytate	Steamed pudding	214	60	128.4
White flour with added sodium phytate	Pastry	214	15	32.1

* These values are calculated from figures given by Widdowson, (1941).

meal flour of 92 per cent. extraction (31 per cent). This is an interesting result because, as seen earlier in this chapter, the distribution of phytate and that of phytase in different parts of the grain are closely similar so that, although there

is much more phytate in wheatmeal flour than in white flour, there is also a much larger amount of phytase.

Widdowson regarded these figures as favouring the view that the endosperm of flour was richer in phytase in relation to its phytate content than had been previously assumed. If, however, instead of percentage hydrolysis, the actual amounts of phytic acid phosphorus which have been hydrolysed in the different breads are considered, the values from the figures given by Widdowson would be 43.4 mg. in white flour, 70 per cent extracted, and 66.3 mg. in wheatmeal, 92 per cent extracted. Thus, although the percentage hydrolysis is lower in the 92 per cent extraction flour, the actual hydrolysis is greater. On the other hand, it is surprising, in view of the much greater amount of phytase in the higher extracted flours, that the increase should be so relatively small. A similar type of result occurred in the present work, when it was found that the phytase of yeast was not as effective in hydrolysing phytate in cereals as in a pure sodium phytate solution. A possible explanation of this result may have been supplied by Hoff-Jørgensen (1947), who recently reported that dried yeast and yeast extracts inhibited the action of cereal phytase. He made no mention of the phytase action of yeast itself. It also seems probable from work in this laboratory that there is an antagonism between yeast phytase and some factor in cereals, and recent results have suggested that, when yeast and bran, both used as sources of phytase, are acting together in the same medium, they are not as potent as the sum of their separate activities. Such an antagonism may be the explanation of the apparent lack of yeast phytase action in some of the tests reported in Table 17.

It is possible, however, now to see the answer to the question raised earlier in this discussion as to what is the fate of phytate in the preparation of bread from wheat and of oatmeal porridge or oatcake from oats, respectively.

In the latter case it has been shown that practically all the phytate of the oats is consumed as such by human beings, whereas, partly because of the elimination of much phytate in milling, and partly because of the destruction of phytate by phytase in cooking, the actual amount of phytate eaten in bread is small as compared with the original amount in the wheat from which it is made. The deduction may probably be made from these facts of the basis for the instinctive desire of those eating oatmeal porridge to add to it a rich supply of calcium such as milk.

Since cereals are the most easily grown and readily transported foodstuffs, it is not surprising that they form the major part of the human diet throughout the world. From the foregoing study it can be seen that, where high cereal diets are normally eaten, three situations as regards the amount of phytate ingested are presented: (1) when the diet contains much phytate, as when oatmeal or corn (maize) is largely eaten and cooked without being otherwise processed; (2) when the diet contains much inositol and inorganic phosphate produced by hydrolysis of phytate in cereals together with some unhydrolysed phytate: for example, when bread made from high extraction flour is eaten or when much corn or millet after fermentation, as in native beer, forms a substantial part of the food; (3) when it contains but little phytate or hydrolysed phytate, as when rice or bread from low extraction flour is eaten.

Since both inositol and phosphate are essential constituents of the body, it is clearly desirable to have good supplies in the food, but only so long as (a) they are absorbable from the gut and available to the body and (b) they are present in a form and under conditions in which they have no such harmful effect in interfering with calcification, as has been shown possible in the case of phytate in Chapter XII. What these conditions are will be seen in the following chapters.

Chapter XIV

FURTHER STUDIES OF THE RELATIVE ANTI-CALCIFYING ACTION OF PHYTATE AND PHOSPHATE UNDER VARYING CONDITIONS

In Chapter XII an account was given of early experiments which established the fact that the rachitogenic effect of oatmeal was due to its phytate content, and it was shown that this effect on calcium metabolism was closely linked up with the amount of calcium in the diet and, of course, with the amount of vitamin D. As regards the relation between phytate and calcium, it was pointed out that the degree of active interference with calcification by a given cereal probably depended on how much phytate and how little available calcium it contained. McCance and Widdowson (1942a), referring to this statement, said that it was too narrow and that, in addition to calcium, other elements in the diet, such as magnesium and iron, should be included. This may be so, but it is undoubted that calcium holds a primary position in this respect.

It was realised at the time of the early work that there were many aspects of the chemistry of phytic acid and its salts about which comparatively little was known. Various tests and chemical estimations were therefore made to ascertain the rate of hydrolysis of the acid and its salts under different conditions (see Chapter XIII) and so to enable the experiments on dogs to be continued with a fuller knowledge of the actual and relative amounts of phytate and phosphate in the diet.

It is proposed now to describe some of the experiments made since 1939, under more controlled conditions than formerly, on the action of phytate in the alimentary canal and on its relation to other factors in the diet. Except

In the latter case it has been shown that practically all the phytate of the oats is consumed as such by human beings, whereas, partly because of the elimination of much phytate in milling, and partly because of the destruction of phytate by phytase in cooking, the actual amount of phytate eaten in bread is small as compared with the original amount in the wheat from which it is made. The deduction may probably be made from these facts of the basis for the instinctive desire of those eating oatmeal porridge to add to it a rich supply of calcium such as milk.

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In Chapter XII an account was given of early experiments which established the fact that the rachitogenic effect of oatmeal was due to its phytate content, and it was shown that this effect on calcium metabolism was closely linked up with the amount of calcium in the diet and, of course, with the amount of vitamin D. As regards the relation between phytate and calcium, it was pointed out that the degree of active interference with calcification by a given cereal probably depended on how much phytate and how little available calcium it contained. McCance and Widdowson (1942a), referring to this statement, said that it was too narrow and that, in addition to calcium, other elements in the diet, such as magnesium and iron, should be included. This may be so, but it is undoubted that calcium holds a primary position in this respect.

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in experiments 14 and 18 to 20, where the effect of additions of Ca and P were being tested, the total Ca and P, and so the Ca:P ratios of the diets of comparable animals, were identical; for instance, when the effect of sodium phytate was being tested, an equivalent amount of P in the form of inorganic phosphate was added to the diet of the control animal. Experiments of this nature were important because the early work (1919–1939) demonstrating the rachitogenic action of cereals, and especially of oatmeal, had been criticised and the results attributed to the Ca:P ratio of the said cereals or to the diets containing them, and not to their phytate content.

It will be remembered that in 1926, before there was any idea of the significance of phytate as a rachitogenic agent, it has been found that cereals boiled with mineral acid or malted usually lost some of their anticalcifying action. The effects of the different stages of malting were more closely studied by M. Mellanby (1929), who showed in her work on teeth that oats lost much of their anticalcifying property when they were steeped, germinated and afterwards ground and allowed to autolyse for about two days, but that steeping and germinating alone did not greatly reduce this effect. A similar result was obtained by Templin and Steenbock (1933) in the case of maize. The reasons for these results were seen in Chapter XIII (Fig. 47) where the effects of various stages of malting on phytate in cereals were described.

On the basis of this work, a further study was made of the respective actions of phytate and the products of its hydrolysis by acid (HCl) and by an enzyme (phytase). In most of the experiments designed for this purpose the cereal preparations with high phytate content caused worse rickets than those in which this substance had been largely converted to inorganic phosphate. In some cases, however, though this was true in the early stages, the condition of

the animals having the inorganic phosphate later deteriorated rapidly and soon approached that of the animals receiving phytate. These irregularities in what appeared to be closely comparable experiments were most perplexing and the time came when it was necessary to see what uncontrolled factors were affecting the results. A detailed survey of such experiments suggested that an important factor might be variations in the initial vitamin D reserves of the animals of different experiments. It appeared that, when the reserves were relatively large, owing to the ingestion of cod-liver oil or other sources of vitamin D during the pre-experimental period, there was good differentiation, but when the reserves were small and were quickly lost, then the animals having inorganic phosphate ultimately developed as severe rickets as those receiving phytate.

This deduction, if true, was obviously of crucial importance because it indicated that, with diets deficient in vitamin D, the relative effects of phytate and phosphate of equal phosphorus content on calcification varied with the amount of vitamin D reserves available for controlling calcium metabolism. The hypothesis was put to the test, and the remainder of this chapter will be devoted to a description of the observations and deductions which resulted from this experimental work. First will be demonstrated the relative rachitogenic effects of oatmeal whose phytate has been hydrolysed to phosphate by boiling with dilute HCl, as compared with the effects of untreated oatmeal, when the reserves of vitamin D at the beginning of the feeding period vary. Afterwards a closer study of the problem by balance metabolism experiments will be described. The discussion at the end of the chapter will give a survey of this complicated problem in its different aspects, and in Chapter XVII attention will be drawn to its significance in relation to man's well-being.

1. Effect of Different Body Reserves of Vitamin D

The puppies used in these experiments received different amounts of vitamin D in the pre-experimental period, and so in some cases would have high and in others lower reserves of this substance. A description is given of the different effects on bone growth and calcification, under these conditions, both when the phytate of the cereal was intact and when it had been wholly or partly hydrolysed to inorganic phosphate. Comparable animals received the same amount of Ca and P in their diets.

(a) Untreated oatmeal (phytate) compared with oatmeal hydrolysed by HCl (phosphate)

EXPERIMENT 7 (HIGH VITAMIN D RESERVES)

The object of describing this experiment is to show the relative effects of oatmeal, before and after acid hydrolysis of its phytate to inorganic phosphate, on bone calcification when the vitamin D reserves are high.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432). Up to 5 ml. of cod-liver oil per puppy per day were added to the food of the litter until the experimental diet was begun at the age of 7 weeks. Thus at the beginning of the experimental period the young animals had a good reserve of vitamin D.

Basal experimental diet:

Oatmeal	80–150 g.
Bread	20 g.
Separated milk powder	25 g.
Lean meat	13–24 g.
Peanut oil	10–18 ml.
NaCl	4.4–8.2 g.
Baker's yeast	3.9–7.2 g.
Ascorbic acid	5 mg.
Cabbage	18–33 g.

Variations in treatment of oatmeal:

No of puppy	
26 (2743)	Untreated (phytate intact).
27 (2744)	Boiled with 1% HCl for 1½ hours, then neutralised (some of phytate hydrolysed to phosphate).*
28 (2745)	Boiled with 1% HCl for 3 hours, then neutralised (some of phytate hydrolysed to phosphate).*
29 (2746)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate).*

* For method of preparation see Appendix II, p. 433.

Age at beginning of experiment: 7 weeks.
Duration of experiment: 11 weeks.

TABLE 19 (Exp. 7)

Effect of boiling oatmeal with HCl on bone calcification when the vitamin D reserves are high

No of puppy	Dietary conditions					Bone results	
	Oatmeal boiled with 1% HCl* (hours)	Phosphorus content of oatmeal eaten (mg. per day)				A/R ratio of femur shaft	Rickets as judged by X-rays at P M ‡
		Phytate		Phosphate†			
		Min	Max	Min	Max		
26	None	222	415	118	222	1.00	8
27	1½	204	382	136	255	1.04	7
28	3	184	345	156	292	1.10	6
29	18	54	100	286	537	1.37	<1

* For method of preparation see Appendix II, p. 433.

† Includes any organic phosphorus which does not react as phytic acid phosphorus.

‡ Rickets graded 1 to 10, the number increasing with the severity of the disease.

The effect on its phytate content of boiling the oatmeal with acid for different periods can be seen by reference to

1. Effect of Different Body Reserves of Vitamin D

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(a) *Untreated oatmeal (phytate) compared with oatmeal hydrolysed by HCl (phosphate)*

EXPERIMENT 7 (HIGH VITAMIN D RESERVES)

The object of describing this experiment is to show the relative effects of oatmeal, before and after acid hydrolysis of its phytate to inorganic phosphate, on bone calcification when the vitamin D reserves are high.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432). Up to 5 ml. of cod-liver oil per puppy per day were added to the food of the litter until the experimental diet was begun at the age of 7 weeks. Thus at the beginning of the experimental period the young animals had a good reserve of vitamin D.

Basal experimental diet:

Oatmeal	80–150 g.
Bread	20 g.
Separated milk powder	25 g.
Lean meat	13–24 g.
Peanut oil	10–18 ml.
NaCl	4.4–8.2 g.
Baker's yeast	3.9–7.2 g.
Ascorbic acid	5 mg.
Cabbage	18–33 g.

Peanut oil	10 ml.
NaCl	1.7–4.4 g.
Baker's yeast	1.5–5.5 g.
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

Variations in treatment of oatmeal:

No of puppy	
30 (2981)	Untreated (phytate intact).
31 (2982)	Boiled with 1% HCl for 6 hours, then neutralised (some of phytate hydrolysed to phosphate).*
32 (2983)	Boiled with 1% HCl for 12 hours, then neutralised (some of phytate hydrolysed to phosphate).*
33 (2985)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate).*

* For method of preparation, see Appendix II, p. 433.

Age at beginning of experiment: 6 weeks.
Duration of experiment: 10 weeks.

TABLE 20 (Exp. 8)

Effect of boiling oatmeal with HCl on bone calcification when the vitamin D reserves are low

No of puppy	Dietary conditions					Bone results			
	Oatmeal boiled with 1% HCl* (hours)	Phosphorus content of oatmeal eaten (mg per day)				A/R ratio of femur shaft	Rickets as judged by X rays‡		
		Phytate		Phosphate†			After 6 weeks on diet	After 8 wks. on diet	After 10 wks on diet (P.M.)
		Min	Max	Min	Max				
30	None	63	167	50	133	0.97	6	7	8
31	6	47	125	66	175	1.01	5	6	7
32	12	35	92	78	208	1.12	2	5	6
33	18	18	48	95	252	1.15	1	5	6

* For method of preparation see Appendix II, p. 433.

† Includes any organic phosphorus which does not react as phytic acid phosphorus

‡ Rickets graded 1 to 10, the number increasing with the severity of the disease.

Fig. 44 (p 257), which is a composite graph based on numerous experiments. Whereas puppy 26 (untreated oatmeal) received 222 to 415 mg. of phytate phosphorus daily at different periods of the experiment, 29 (18 hours' treatment) received only 54 to 100 mg. and the remaining 168 to 315 mg. daily as phosphate, practically all in inorganic form.

The A/R ratios and radiographs of puppies 26 and 29 after 11 weeks on diet are shown in Table 19 and Plate XXII, *a* and *b*. It will be seen that, under these particular conditions, when the vitamin D reserves of the animals are fairly high (Cod-liver oil in the pre-experimental food), the rickets-producing effect of untreated oatmeal containing phytate is much greater than that of oatmeal whose phytate has been largely converted to phosphate, although the total P content of the cereal is unchanged. Boiling the oatmeal with dilute acid for 1½ and 3 hours, respectively, has made little difference to its phytate content, to the degree of rickets, or to the A/R ratios of the femur shafts.

EXPERIMENT 8 (LOW VITAMIN D RESERVES)

Object. This experiment is included in order to demonstrate the relative effect of oatmeal, before and after acid hydrolysis of its phytate content, on bone calcification when the vitamin D reserves are low.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) This series of puppies received no cod-liver oil or other form of vitamin D when fed independently of the mother. Thus it can be assumed that the reserves of vitamin D at the beginning of the experimental period were lower than those of the animals of experiment 7.

Basal experiment diet:

Oatmeal	30–80 g.
Wheatmeal bread	20–50 g
Separated milk powder	20 g.
Lean meat	15 g.

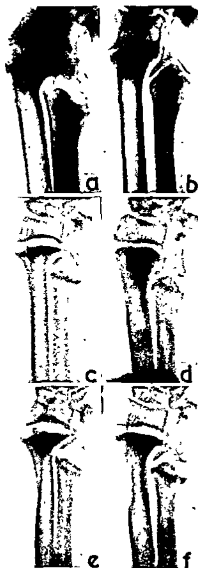


PLATE XXII

The relative amount of phytate phosphorus in these diets was rather less than in the previous experiment, due to the lower phytate content of the original oatmeal. Plate XXII, *c* and *d*, shows the degree of rickets in puppies 30 (high phytate) and 33 (low phytate) after six weeks of the experimental diet. It will be seen that at this point 30 has the worse rickets, but in radio-graphs taken only 2 weeks later (Plate XXII, *e and f*), the differentiation was disappearing and 33 was rapidly developing severe rickets. This was the type of result which, until the significance of the vitamin D reserves was realised, had been so disconcerting.

The A/R ratios of the femur shafts in this experiment, although still indicating slight improvement in calcification in the animals receiving hydrolysed phytate, do not show as big differences as were seen in experiment 7, the animals of which had a larger reserve of vitamin D.

These results, typical of the older experiments, support the suggestion that the differences between the rickets-pro-

PLATE XXII

Experiment 7. a and b. Radiographs of forepaws at end of experiment, showing relative rickets-producing effect of phytate and phosphate in the presence of high vitamin D reserves.

a. Puppy 26 Untreated oatmeal (phytate intact).

b. Puppy 29 Oatmeal which had been boiled for 18 hours with 1% HCl (most of phytate hydrolysed to phosphate).

Note: Puppy 26 had severe rickets whereas puppy 29 was still normal after 11 weeks.

Experiment 8. c to f. Radiographs of forepaws showing relative rickets-producing effect of phytate and phosphate in the presence of low vitamin D reserves.

c and e. Puppy 30. Untreated oatmeal (phytate intact).

c, 6 weeks on diet.

e, 8 weeks on diet.

d and f. Puppy 33. Oatmeal which had been boiled for 18 hours with 1% HCl (most of phytate hydrolysed to phosphate).

d, 6 weeks on diet.

f, 8 weeks on diet.

Note: After 6 weeks on the experimental diets (see c and d) puppy 33 (phosphate) had less severe rickets than puppy 30 (phytate), but 2 weeks later the condition of the two puppies was more similar (see e and f).

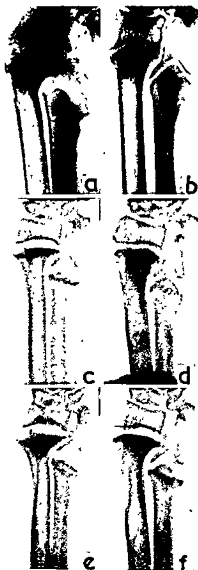


PLATE XXII

ducing effects of untreated oatmeal (phosphorus present mainly as phytate) and treated oatmeal (most of phytate hydrolysed to phosphate) were modified by differences in the bodily stores of vitamin D at the beginning of the experiment.

In these early experiments attention was focussed on the condition of the bones, as judged by X-ray examination at the beginning and during the course of the experiments and by determining the mineral ash content and the A/R ratio of one or more bones at the end of the period. It seemed essential at this stage to extend the investigation to the study of the absorption of Ca and P from the intestines and the excretion of these substances in both the faeces and the urine under the varying conditions, for it was likely that any difference there might be between the effects of phytate and phosphate on calcium and phosphorus metabolism would be at least partially, and possibly wholly, shown in the alimentary canal. Thus in all the following experiments both the end results as regards bone calcification and the course of Ca and P balances during the experimental period were investigated. In the case of P metabolism, both phytate and total phosphorus were determined. The animals were put at intervals of 2 to 4 weeks in metabolism cages for periods of 2 and later 3 days, the faecal excretion of the period being marked at the beginning and the end by giving carmine in the food and so colouring the stools. The total urine passed during a 2- or 3-day period was also collected. Any dogs suspected of eating excreta were muzzled when in the metabolism cages and care was taken to ensure as prompt removal as possible of faeces from the cages. This was usually carried out without difficulty, but in some cases, due to loose stools, shaggy coats, or excessive activity of the dogs, there must have been some loss. For this reason the curves representing Ca and P retentions or excretions must be

considered as a whole and too much emphasis ought not to be placed on individual estimations. When severe rickets occurred it was sometimes impossible to obtain true samples towards the end of the experiment, partly because the animals did not always eat their food promptly and partly because of their immobility. In most of these cases the metabolic tests were perforce stopped, although they might be continued for other members of the litter.

(b) Untreated oats (phytate) compared with germinated and autolysed oats (phosphate)

EXPERIMENT 9 (HIGH AND MEDIUM VITAMIN D RESERVES)

The object of this experiment was to test the effect on bone calcification and calcium retention in the presence of different vitamin D reserves of oats, before and after the phytate of the cereal had been hydrolysed by germination and autolysis.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The first pair of puppies (34 and 35) had a daily addition of 2.5 ml. of cod-liver oil for 4 weeks prior to the experiment and would have good bodily reserves of the vitamin. The second pair (36 and 37) received no additional vitamin D or cod-liver oil and therefore they would have lower reserves of the vitamin when the experiment began.

Basal experimental diet:

Oats	20–200 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i.u.

(Ca content: initial 407 mg., final 565 mg.)
(P content: initial 326 mg., final 904 mg.)

ducing effects of untreated oatmeal (phosphorus present mainly as phytate) and treated oatmeal (most of phytate hydrolysed to phosphate) were modified by differences in the bodily stores of vitamin D at the beginning of the experiment.

In these early experiments attention was focussed on the condition of the bones, as judged by X-ray examination at the beginning and during the course of the experiments and by determining the mineral ash content and the A/R ratio of one or more bones at the end of the period. It seemed essential at this stage to extend the investigation to the study of the absorption of Ca and P from the intestines and the excretion of these substances in both the faeces and the urine under the varying conditions, for it was likely that any difference there might be between the effects of phytate and phosphate on calcium and phosphorus metabolism would be at least partially, and possibly wholly, shown in the alimentary canal. Thus in all the following experiments both the end results as regards bone calcification and the course of Ca and P balances during the experimental period were investigated. In the case of P metabolism, both phytate and total phosphorus were determined. The animals were put at intervals of 2 to 4 weeks in metabolism cages for periods of 2 and later 3 days, the faecal excretion of the period being marked at the beginning and the end by giving carmine in the food and so colouring the stools. The total urine passed during a 2- or 3-day period was also collected. Any dogs suspected of eating excreta were muzzled when in the metabolism cages and care was taken to ensure as prompt removal as possible of faeces from the cages. This was usually carried out without difficulty, but in some cases, due to loose stools, shaggy coats, or excessive activity of the dogs, there must have been some loss. For this reason the curves representing Ca and P retentions or excretions must be

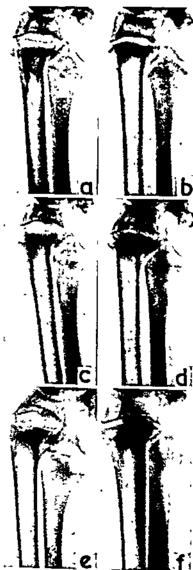


PLATE XXIII

Variations in treatment of oats:

No of puppy	
34 (3225)	Ground and boiled (phytate intact).
35 (3227)	Germinated, minced and autolysed (phytate hydrolysed to phosphate).*
36 (3224)	Ground and boiled (phytate intact).
37 (3226)	Germinated, minced and autolysed (phytate hydrolysed to phosphate).*

* For method of preparation see Appendix II, p. 433.

Age at beginning of experiment: 6 weeks.

Duration of experiment: 17 weeks.

Radiographs (Plate XXIII, *a*, *b*, *c* and *d*) indicate that when the body has good reserves of vitamin D at the beginning of the experiment, as in puppies 34 and 35 (Figs. *a* and *b*), the differentiation between the rickets-producing effects of phytate and phosphate is greater than when the

PLATE XXIII

Experiment 9 a–d. Radiographs showing the relative rickets-producing effect in the presence of high and medium vitamin D reserves respectively, of oats before and after part of their phytate had been hydrolysed by germination and autolysis.

a Puppy 34. Oats before treatment (phytate intact); high vitamin reserves

b. Puppy 35. Oats after treatment (phytate hydrolysed to phosphate); high vitamin reserves.

c Puppy 36. Oats before treatment (phytate intact); medium vitamin reserves.

d. Puppy 37 Oats after treatment (phytate hydrolysed to phosphate); medium vitamin reserves.

Note: there is a greater difference in the degree of rickets between (a) and (b) (high vitamin reserves) than between (c) and (d) (medium vitamin reserves) after 17 weeks on diet.

Experiment 10. e and f. Radiographs showing the rickets-producing effect in the presence of high vitamin D reserves, of oats, before and after their phytate had been hydrolysed by germination and autolysis.

e. Puppy 38. Oats before treatment (phytate intact); high vitamin D reserves.

f. Puppy 39 Oats after treatment (phytate hydrolysed to phosphate) high vitamin D reserves.

Note: The high vitamin D reserves have increased the difference between the degrees of rickets produced by phytate (e) with bad rickets and phosphate (f) which is nearer normal.

tate of oats intact) and the second puppies 35 and 37 (phytate of oats converted to phosphate), the amount of calcium retained by the phosphate pair remaining at a higher level than that retained by the phytate pair. In each pair the puppy which originally had high reserves of vitamin D retained slightly more Ca than that with the medium reserves.

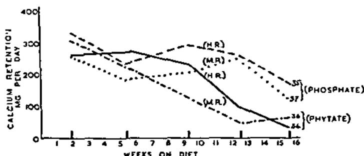


FIG. 51 (Exp. 9). Relative effects of untreated and of germinated and autolysed oats on calcium retention in the presence of high and medium reserves of vitamin D. ⊥ Balance tests on these dates.

Note· In the presence of both high (H.R.) and medium (M R) vitamin D reserves the Ca retention of puppies 34 and 36, having untreated oats (phytate), was lower than that of puppies 35 and 37, receiving germinated, minced and autolysed oats (phytate converted to phosphate).

There were in this litter two other puppies, which were kept on the pre-experimental diet containing cod-liver oil until they were 10 weeks old, instead of 6 weeks, in order to increase their reserves of vitamin D. They were then put on experimental diets, one having as the cereal moiety oats before germination and the other oats after germination and autolysis. The experimental period (see Exp. 10) lasted for the same length of time as that of the series described in experiment 9, but as the two animals were older when put on diet they consumed more food.

reserves are lower, as in puppies 36 and 37 (see *c* and *d*). Fig. 51, representing the daily calcium retentions of the four puppies, shows that when they are compared according to their vitamin D reserves there is but little difference between the phytate and the phosphate animal of each

TABLE 21 (Exp. 9)

Effect of untreated oats (phytate) compared with germinated and autolysed oats on bone calcification when vitamin D reserves are high and medium

No. of puppy	Vitamin D reserves	Dietary conditions					Bone results	
		Treatment of oats	Phosphorus content of oats eaten (mg. per day)				A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.†
			Phytate		Phosphate*			
			Min.	Max.	Min.	Max.		
34	High	Ground and boiled	28	280	33	330	1.05	5
35	High	Germinated, minced and autolysed	0	0	61	610	1.19	2
36	Medium	Ground and boiled	28	280	33	330	1.03	6
37	Medium	Germinated, minced and autolysed	0	0	61	610	1.15	4

* Includes any organic phosphorus which does not react as phytic acid phosphorus.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

pair up to the sixth week of the experimental feeding, when they are eating only relatively little oats and the amount of phytate consumed is still small. From this point, however, as the phytate of the diet increases there is a division into two groups, the first including puppies 34 and 36 (phy-

phytate also brings about an increase in Ca retention, except during the first few weeks of the experiment when the amount of cereal consumed, and therefore P intake, is small (Fig. 52).

In view of the above results, from which it appeared that phytate in oatmeal and oats lost some of its rachitogenic effect after hydrolysis to inorganic phosphate and

TABLE 22 (Exp. 10)

Effect of hydrolysing the phytate of oats by germination and autolysis on bone calcification when the vitamin D reserves are high

No of puppy	Vitamin D reserves	Dietary conditions: Treatment of oats	Phosphorus content of oats eaten (mg per day): Phytate Min.	Phytate Max	Phosphate† Min.	Phosphate† Max	Bone results: A/R ratio of femur shaft	Rickets as judged by X-rays at P.M ‡
38	High	Ground and boiled	70	350	83	413	0.89	8
39	High	Germinated, minced and autolysed*	0	0	153	763	1.06	3

* For method of preparation see Appendix II, p. 433.

† Includes any organic phosphorus which does not react as phytic acid phosphorus.

‡ Rickets graded 1 to 10, the number increasing with the severity of the disease.

that the relative effects of these two forms of P in the diet were in some way determined by the presence in or absence from the body of vitamin D, it was decided to make a more complete study of the problem. Instead of giving cereals containing phytate, this substance was prepared as the neutral sodium salt and given to puppies either as such or, after hydrolysis by yeast, as inorganic phosphate. It thus became possible to get a clearer view of the relative action of

EXPERIMENT 10

The object of this experiment was to test the relative effect of oats, before and after the phytate has been hydrolysed by germination and autolysis, on bone calcification and calcium retention when the vitamin D reserves of the animals were high.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The puppies received a daily addition of 2.5 ml. of cod-liver oil for eight weeks before the experimental diet was begun and would, therefore, presumably have high reserves of vitamin D.

Basal experimental diet:

Oats	50–250 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i.u.

(Ca content: initial 432 mg., final 609 mg.)
(P content: initial 424 mg., final 1064 mg.)

Variations in treatment of oats:

No of puppy	
38 (3229)	Ground and boiled (phytate intact).
39 (3228)	Germinated, minced and autolysed* (phytate hydrolysed to phosphate).

* For method of preparation see Appendix II, p. 433.

Age at beginning of experiment: 10 weeks.
Duration of experiment: 17 weeks.

Under the conditions of this experiment, when vitamin D reserves are high, converting the phytate of the diet to inorganic phosphate results in a reduction of the rachitogenic effect (Plate XXIII, *e* and *f*). This treatment of the

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) From the age of 4 to 7 weeks puppies 40 and 41 were each given 500 i.u. vitamin D_2 daily. It was certain, therefore, that both had *substantial stores of the* vitamin at the beginning of the experiment. Puppies 42 and 43, on the other hand, although receiving the same low vitamin D basal diet, had no vitamin D_2 supplement, so that their stores were smaller.

Basal experimental diet:

White flour	35–145 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	*10 ml.*
NaCl	1% of cereal
Baker's yeast	10% of cereal
Ascorbic acid	*5 mg.*
Vitamin A acetate	1000 i.u.

(Ca content: initial 406 mg., final 421 mg.)
(P content initial 301 mg , final 393 mg.)

Daily additions to basal diet:

No of puppy	Mg. phosphorus per 100 g. flour Na phytate	Na phosphate
40 (3170)	250	10
41 (3169)	0	260
42 (3168)	250	10
43 (3167)	0	260

Age at beginning of experiment: 7 weeks.
Duration of experiment: 23–24 weeks.

All four puppies receive the same total amount of phosphorus daily, but the quantity given to 41 and 43 in excess of that present in the basal diet is in the inorganic form, whereas the extra given to 40 and 42 is mainly phytate phosphorus. It will be seen from Fig. 53 that the calcium retention of 40 and 41, both having a comparatively large store of vitamin D at the beginning of the experiment, remains high for the first 9 weeks. There is, how-

these substances on Ca absorption and bone calcification uncomplicated by other unknown dietetic factors.

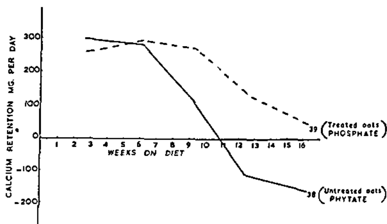


FIG. 52 (Exp. 10). Effect of hydrolysing the phytate of oats by *germination and autolysis on calcium retention when vitamin* D reserves are high.

Note: Oats with intact phytate (38) caused a much smaller retention of Ca than oats the phytate of which had been hydrolysed (by germination and autolysis) to phosphate (39) when the body reserves of vitamin D were high.

(c) *Sodium phytate prepared from commercial phytin compared with sodium phosphate*

EXPERIMENT 11 (HIGH AND MEDIUM VITAMIN D RESERVES)

The object of this experiment was to see in the first place whether the results obtained in experiments 7–10, in which treated and untreated oatmeal and oats were the variable factors, could be repeated with phytate and inorganic phosphate. It was also desired to determine whether there was a difference in the amounts of Ca and P absorbed from the gut when *these two salts were used and, if so,* whether this difference persisted when the vitamin D reserves were exhausted.

ever, during this period a real difference between the amount retained by the two animals, 41 (receiving phosphate) having a consistently higher retention than 40 (phytate). After 11 weeks of the diet it would appear that the vitamin D stores of these two puppies are greatly reduced and the power to retain calcium is being rapidly lost. In puppies 42 and 43, whose vitamin D reserves are known to have been smaller at the beginning of the experimental period, the Ca retention soon begins to fall and there is little difference between the absorption of Ca in the phytate and phosphate animals in this respect after 3 weeks.

The radiographs of these animals show that the high vitamin D reserves of puppies 40 and 41 have protected them against rickets until the thirteenth week of the experiment, at which time 42 and 43, with the smaller reserves, have severe rickets. At the end of the experimental period (i.e. after approximately 24 weeks) the disease has developed in the previously rickets-free pair but to a less severe extent. In both pairs the puppy receiving phytate is more rachitic than that receiving phosphate, but in the pair with only moderate vitamin D reserves the definite differentation observed after 10 weeks of the experiment has been gradually reduced.

The results of this experiment, therefore, lend support to the hypothesis upon the basis of which it was carried out. It suggests that (1) when vitamin D is present in the body, even when there is none in the food, phytate prevents the absorption and retention of calcium to a greater extent than phosphate of equal phosphorus content; and (2) when the body has no reserves of the vitamin and there is none in the food, calcium absorption is very small in any case, and phosphate is probably as potent as phytate in further reducing its absorption.

Although in this experiment there is a definite difference between the A/R ratios of the bones of animals with and

TABLE 23 (Exp. 11)

Relative effects of phytate and phosphate on bone calcification when vitamin D reserves are high and medium

No of puppy	Vitamin D reserves	Dietary conditions				Bone results			
		Phosphorus *added* to basal diet (mg. per day)				A/R ratio of femur shaft	Rickets as judged by X rays†		
		Na phytate		Inorganic phosphate*			After 10 wks. on diet	After 13 wks. on diet	At P.M
		Min	Max	Min	Max.				
40	High	88	363	3	14	1.31	0	0	3
41	High	0	0	91	377	1.35	0	0	2
42	Medium	88	363	3	14	0.74	8	9	10
43	Medium	0	0	91	377	0.66	4	6	8

* Na phytate hydrolysed by phytase of yeast.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

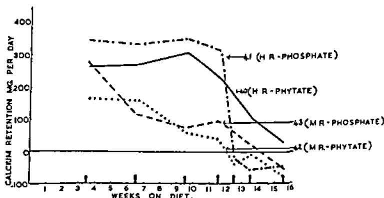


FIG. 53 (Exp. 11) Relative effects of phytate and phosphate on calcium retention in the presence of high and medium reserves of vitamin D

Note: 1. When the vitamin D reserves were high (H.R.) the Ca retention of puppy 40 (phytate) was lower than that of puppy 41 (phosphate prepared from phytate) but with the depletion of reserves Ca retentions of both puppies fell;

2. When the vitamin D reserves were lower (M.R.) there was but little difference in the Ca retentions of puppies 42 (phytate) and 43 (phosphate prepared from phytate).

there should have been only moderate reserves of the vitamin in all the animals.

Basal experimental diet:

Oatmeal	35–140 g.
Bread.	20 g.
Separated milk powder.	30 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl.	4.8% of cereal
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i.u.

(Ca content: initial 362 mg, final 414 mg.)
(P content: initial 436 mg., final 883 mg.)

Variations in treatment of oatmeal:

No. of puppy	
44 (3207)	Untreated (phytate intact).
45 (3209)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate).*
46 (3210)	Untreated (phytate intact).
47 (3212)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate).*

* For method of preparation see Appendix II, p. 433.

Daily additions to basal diet:

No. of puppy	
44	20 i.u. vitamin D_2
45	20 i.u. vitamin D_2
46	None
47	None

Age at beginning of experiment: 7 weeks.

Duration of experiment: 46 and 47, 17½ weeks; 44 and 45, 19½ weeks.

In this experiment oatmeal was the source of phytate and phosphate.

Although all the animals in this litter receive the same

without vitamin D reserves, there is not much difference in either pair between the animals which received phytate and phosphate P. In 42 and 43 (low vitamin D reserves) it would not be expected, but it is considered surprising that there is no difference between 40 and 41.

Closer examination of Ca retentions, mineral content and A/R ratios of bones revealed that these three indexes of calcium metabolism, although related in a general way, have different significance when used in relation to bone formation and calcification. A/R ratios are a useful indication of the structure and degree of calcification of the bone, whereas the Ca retention and the mineral content of bone, which are usually in agreement, may give no indication of the bone structure. (See full discussion, p. 346.)

It was next decided to see whether phytate and phosphate, both in oatmeal and as the Na salts prepared from commercial phytin, had the same relative effects on the absorption and utilisation of Ca when animals received no vitamin D and 20 i.u. daily respectively.

2. Effect of Small Amounts of Dietary Vitamin D.

(a) *Untreated oatmeal (phytate) compared with oatmeal hydrolysed by HCl (phosphate)*

EXPERIMENT 12

The object of this experiment was to determine the relative effect of oatmeal, both before and after the phytate had been converted into phosphate by hydrolysis with HCl, on bone calcification and on calcium metabolism. The tests were made both in the presence and in the absence of dietary vitamin D.

Pre-experimental diet. (For maternal diet see Appendix II, p. 423.) When fed independently of the mother, this series of puppies received no cod-liver oil or other source of vitamin D. Thus at the beginning of the experiment

there should have been only moderate reserves of the vitamin in all the animals.

Basal experimental diet:

Oatmeal	35–140 g.
Bread	20 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	4.8% of cereal
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate.	1000 i.u.

(Ca content: initial 362 mg , final 414 mg.)
(P content: initial 436 mg., final 883 mg.)

Variations in treatment of oatmeal:

No. of puppy	
44 (3207)	Untreated (phytate intact).
45 (3209)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate).*
46 (3210)	Untreated (phytate intact).
47 (3212)	Boiled with 1% HCl for 18 hours, then neutralised (most of phytate hydrolysed to phosphate) *

* For method of preparation see Appendix II, p. 433.

Daily additions to basal diet:

No. of puppy	
44	20 i.u. vitamin D_2
45	20 i.u. vitamin D_2
46	None
47	None

Age at beginning of experiment: 7 weeks.

Duration of experiment: 46 and 47, 17½ weeks; 44 and 45, 19½ weeks.

In this experiment oatmeal was the source of phytate and phosphate.

Although all the animals in this litter receive the same

total amount of P in the food, it will be seen from Table 24 that the P of 45 and 47 (acid-treated oatmeal) is mostly in the inorganic form, whereas that of 44 and 46 contains much phytate. The Ca balance results are given in Fig. 54, which shows that in 46 and 47, neither of which has vitamin D and both of which develop severe rickets (as judged by X-rays), the Ca retention falls throughout the experiment and the phytate reduces the Ca balance to a

TABLE 24 (Exp. 12)

Relative effects of phytate and phosphate on bone calcification with and without dietary vitamin D_2 (20 i.u.)

No. of puppy	Dietary conditions						Bone results	
	Oatmeal boiled with 1% HCl (hours)	Phosphorus in oatmeal eaten				Vitamin D_2, i.u.	A/R ratio of femur shaft	Rickets as judged by X rays at P.M.†
		Phytate		Phosphate*				
		Min.	Max.	Min.	Max.			
44	0	80	380	47	220	20	1.52	0
45	18	23	113	104	487	20	1.52	0
46	0	80	380	47	220	0	0.795	10
47	18	23	113	104	487	0	0.95	9

* Includes any organic phosphorus which does not react as phytic acid phosphorus.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

greater extent than the phosphate. Vitamin D protects puppies 44 and 45 against rickets, but although both maintain a good positive calcium balance, puppy 44, receiving phytate P, has a lower retention than 45, receiving phosphate.

This experiment, therefore, indicates that, even in the presence of sufficient dietary vitamin D_2 to protect the animals against rickets, phytate exerts a greater inhibiting

effect on Ca absorption than phosphate, thereby lowering retention, whereas in the absence of the vitamin the absorption of Ca is small and irregular whether phytate or phosphate is given.

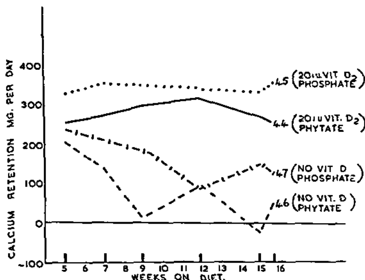


FIG 54 (Exp 12). Relative effects of untreated and HCl treated oatmeal on calcium retention in the absence of dietary vitamin D and in the presence of 20 i.u. daily.

Untreated oatmeal (phytate) (44 and 46) exerted a greater inhibiting action on calcium absorption than treated oatmeal (phosphate) (45 and 47) both in the presence of dietary vitamin D_2 (44 and 45) and in its absence (46 and 47).

(b) Sodium phytate (from phytin) compared with sodium phosphate (sodium phytate hydrolysed by yeast)

EXPERIMENT 13

Object. To determine the relative effects of sodium phytate and sodium phosphate (prepared from phytate by hydrolysis with yeast phytase) on bone calcification and

total amount of P in the food, it will be seen from Table 24 that the P of 45 and 47 (acid-treated oatmeal) is mostly in the inorganic form, whereas that of 44 and 46 contains much phytate. The Ca balance results are given in Fig. 54, which shows that in 46 and 47, neither of which has vitamin D and both of which develop severe rickets (as judged by X-rays), the Ca retention falls throughout the experiment and the phytate reduces the Ca balance to a

TABLE 24 (Exp. 12)

Relative effects of phytate and phosphate on bone calcification with and without dietary vitamin D_2 (20 i.u.)

No of puppy	Dietary conditions						Bone results	
	Oatmeal boiled with 1% HCl (hours)	Phosphorus in oatmeal eaten				Vitamin D_2, i u.	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M †
		Phytate		Phosphate*				
		Min.	Max.	Min	Max.			
44	0	80	380	47	220	20	1.52	0
45	18	23	113	104	487	20	1.52	0
46	0	80	380	47	220	0	0.795	10
47	18	23	113	104	487	0	0.95	9

* Includes any organic phosphorus which does not react as phytic acid phosphorus.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

greater extent than the phosphate. Vitamin D protects puppies 44 and 45 against rickets, but although both maintain a good positive calcium balance, puppy 44, receiving phytate P, has a lower retention than 45, receiving phosphate.

This experiment, therefore, indicates that, even in the presence of sufficient dietary vitamin D_2 to protect the animals against rickets, phytate exerts a greater inhibiting

TABLE 25 (Exp. 13)

Relative effects of phytate and phosphate on bone calcification with and without dietary vitamin D_2 (20 i.u.)

No. of puppy	Dietary conditions					Bone results	
	Phosphorus added to basal diet (mg. per day)				Vitamin D_2, i.u.	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.†
	Na phytate		Na phosphate*				
	Min.	Max.	Min.	Max.			
48	62	400	3	16	20	1.06	0
49	0	0	65	416	20	1.60	0
50	62	400	3	16	None	0.99	9
51	0	0	65	416	None	0.91	9

* Na phytate hydrolysed by phytase of yeast. For details of method, see Appendix II, p. 435.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

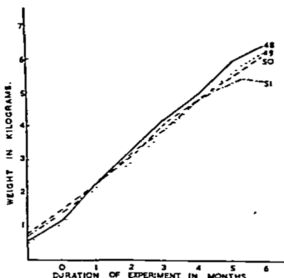


Fig. 55 (Exp. 13). Weight increases of a litter of 4 puppies

These weight curves are typical of those of the litters of puppies described in this chapter.

on calcium metabolism in the presence and absence of dietary vitamin D.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The mother of the litter received cod-liver oil during pregnancy and the first 4 weeks of lactation but the food given to the puppies contained no cod-liver oil or other form of vitamin D.

Basal experimental diet:

White flour	25–160 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1% of cereal
Baker's yeast	10% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i.u.

(Ca content: initial 392 mg., final 434 mg.)
(P content: initial 295 mg., final 450 mg.)

Daily additions to basal diet:

No of puppy	Mg. phosphorus per 100 g. flour Na phytate	Na phosphate	Vitamin D_2, i u.
48 (3156)	250	10	20
49 (3157)	0	260	20
50 (3159)	250	10	None
51 (3158)	0	260	None

Age at beginning of experiment: 6½ weeks.
Duration of experiment: 23 weeks.

Fig. 55 shows the weight increases of these puppies; they are typical of those in the experiments described in this chapter.

These animals were periodically tested for calcium and phosphorus retention and the findings as regards Ca are given in Fig. 56, which shows that (1) when the diet contains a small amount of vitamin D_2, phytate interferes with Ca retention more than inorganic phosphate of the same P content, and (2) when the body and diet are devoid

times it has been present as inorganic phosphate and sometimes partly as phytate.

The results of experiments 7–13 show that:

(1) In the presence of vitamin D, either in the diet or in the body as reserves, phytate reduces Ca absorption from the gut more than does inorganic phosphate of equal P content.

(2) As the vitamin D stores of the body are used up, Ca absorption is always greatly diminished in the presence of either phytate or phosphate, phytate usually, though not in all cases, proving the more powerful in this respect.

(3) So far as is indicated by Ca retention and radiographs of the bones, the effects of giving a high phytate-containing cereal such as oatmeal, before and after treatment which converts much of its phytate to inorganic phosphate, can be repeated by adding sodium phytate and sodium phosphate respectively to diets of low phytate content.

The time and rate at which the Ca retention of D-deficient animals falls varies from experiment to experiment. This irregularity is largely due to differences in and rates of loss of body stores of the vitamin and can be largely controlled by regulating the vitamin D intake in the pre-experimental period.

Having demonstrated that, in the presence of vitamin D, phytate has a specific action in reducing Ca absorption, it now seems desirable to compare the effect of adding phytate and phosphate respectively to diets of relatively low P content. The previous experiments, owing to their form, have not disclosed whether inorganic phosphate, in the amounts given, has any depressing effect on Ca absorption from the gut, but the results suggest that, when there is sufficient vitamin D available, additional phosphate has comparatively little effect, whilst phytate definitely causes

of vitamin D_2, there is but little difference between phytate and phosphate in their effects on Ca retention.

The A/R ratios of the femur shafts clearly show that in the presence of dietary vitamin D_2 phytate as compared with phosphate has the effect of reducing the quality of the bone formed (cf. 48 and 49), whereas in the absence

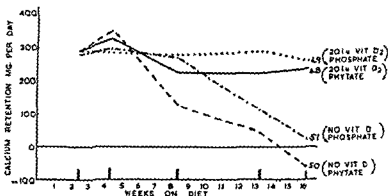


FIG. 56 (Exp. 13). Relative effects of phytate and phosphate on calcium retention in the absence of vitamin D and in the presence of 20 i u. daily

Note: 1. In the presence of dietary vitamin D_2 retention of Ca was lower with phytate (48) than with phosphate (49);

2. In the absence of dietary vitamin D_2 the power to retain Ca was soon reduced in both animals irrespective of the form of P ingested [phytate animal (50) about 2 weeks before phosphate (51)].

of the vitamin the two salts have more similar effects (cf. 50 and 51).

In all the foregoing experiments the dietary Ca:P ratio has been low at the beginning and has fallen during the course of the feeding period as the consumption of cereal has increased. Although the amounts and ratios of Ca and P have varied from experiment to experiment, they have been identical for all puppies of a litter at any given time. The form of the phosphorus, however, has varied; some-

TABLE 26 (Exp. 14)

Effect of increasing the dietary phosphorus by additions of phytate and phosphate on bone calcification

No. of puppy	Dietary conditions: Phosphorus added to basal diet (mg per day): Na phytate, Min.	Na phytate, Max.	Na phosphate*, Min	Na phosphate*, Max.	Vitamin D_2, i u	Bone results: A/R ratio of femur shaft	Rickets as judged by X-rays†: After 7 wks on diet	After 11 wks on diet	At P.M.
52	50	400	2	16	2	0.98	2	6	8
53	0	0	52	416	2	1.08	1	3	5
54	0	0	0	0	2	1.23	0	1	3

* Na phytate hydrolysed by phytase of yeast

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

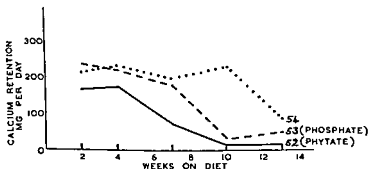


FIG. 57 (Exp. 14). Relative effects of reducing the Ca:P ratio of the diet by additions of phytate or phosphate P on Ca retention.

Note: (1) when, in addition to a trace (2 i u. daily) of vitamin D_2 there were also body reserves, lowering the Ca:P ratio by the addition of phytate (52) reduced Ca retention to a greater extent than a similar change in the ratio brought about by phosphate (53);

(2) later (after 10 weeks) when the vitamin D reserves were depleted the effects of the two forms of P were more alike;

(3) the addition of P in either form appeared to hasten the loss of vitamin D reserves, as indicated by diminution of Ca absorption, and phytate probably did this to a greater extent than phosphate.

a large reduction in the Ca absorbed; on the other hand, when the vitamin reserves are depleted the two substances probably have more equal depressing effects on Ca absorption.

The next experiment is designed to test this point. Two puppies are given diets of a lower Ca:P ratio than the control, one receiving an addition of P as phytate and the other an equal amount as phosphate. All three animals have a trace of dietary vitamin D_2 to supplement the body reserves but not sufficient to prevent rickets.

3. Effect of Increasing the Dietary Phosphorus by Addition of Phytate and Phosphate

EXPERIMENT 14

Pre-experimental diet. When the puppies were fed separately from the mother they received no cod-liver oil or other source of vitamin D.

Basal experimental diet:

White flour	20–160 g.
Separated milk powder	20 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1% of cereal
Baker's yeast	10% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 267 mg., final 292 mg.)
(P content initial 230 mg., final 410 mg.)

Daily additions to basal diet:

No of puppy	Mg. phosphorus per 100 g flour Na phytate	Na phosphate*	Vitamin D_2, u
52 (3129)	250	10	2
53 (3130)	0	260	2
54 (3131)	0	0	2

Age at beginning of experiment: 6½ weeks.
Duration of experiment: 15½ weeks.

agent has been based on rat experiments in which diets with a high-Ca, low-P ratio are used and in which P is without doubt the limiting factor as regards calcification. In such experiments the addition of P to the diet improves both Ca and P absorption and bone calcification. In the present experiment, however, where Ca is the limiting factor, additions of P either as phytate or phosphate reduce Ca absorption, as has been shown above, but result in an increased absorption of P. Phosphate, which has the smaller depressing effect on Ca absorption, increases the P absorption to a greater extent than phytate. Thus after 4 weeks, when there are still body reserves of vitamin D, the addition of phytate increases P absorption from 267 mg. (54) to 393 mg. (52) daily, and the addition of phosphate increases it from 267 mg. (54) to 495 mg. (53) daily. Later (13 weeks), when the vitamin reserves are very low, the P absorptions are 191 mg. (54, no extra P), 385 mg. (52, phytate), and 589 mg. (53, phosphate). Most of the additional P absorbed from the high-P diets is excreted via the urine, so that the amounts of P retained by the three animals are approximately the same.

This experiment therefore suggests that:

(1) 2 i.u. of vitamin D daily do not prevent the depletion of this vitamin, since all three animals so fed lose the power to maintain Ca absorption.

(2) Adding equal amounts of phytate and phosphate, respectively, to the diets of litter-mate puppies, and thereby lowering the Ca:P ratios of their food to the same extent, has widely different effects on their Ca retention as long as sufficient vitamin D is available.

(3) The addition of phytate to the diet hastens the onset of rickets and increases its severity to a greater degree than the addition of an equal amount of P as phosphate.

(4) The addition of either type of P appears to hasten

Fig. 57 shows that during the early part of the experiment, when all three animals have some reserves of vitamin D, the addition to the diet of phosphate (53), and incidentally the reduction of the Ca:P ratio, has but little effect on Ca retention, whereas the addition of the same amount of P as phytate (52), and the same alteration in the Ca:P ratio, has a definite effect. After 10 weeks of the experiment the differentiation between puppies 52 and 53 is disappearing, phytate and phosphate now behaving similarly in that both reduce the Ca retention. After a further 3 weeks, at the time of the last metabolic test, all the animals have greatly reduced powers of Ca absorption, but the two receiving diets with the lower Ca:P ratios still retain less than the control animal (54).

Fig. 57 also shows that the 2 i.u. of vitamin D given daily to each of the animals are not sufficient either to maintain the full power of Ca absorption or even to conserve the body stores of the vitamin. The Ca retention curves of the three animals fall sharply but at different times. Thus the control animal (54) begins to lose its power to retain Ca after 10 weeks, the phosphate animal (53) after 7 weeks, and the phytate animal (52) after 4 weeks. It would appear that the addition of either type of P hastens the removal of stored vitamin D and that phytate does so more rapidly than phosphate.

X-ray examination of the bones shows that after 7 weeks of the experimental feeding there is definite rickets in puppy 52 (phytate), whilst 53 (phosphate) is still nearly normal and 54 is without doubt normal from this point of view. From this time onwards the disease develops in the three animals, the two with additional P developing more severe rickets than the control (54), while puppy 52 (phytate) has worse rickets than 53 (inorganic phosphate).

Most of the criticism of phytate as an anticalcifying

agent has been based on rat experiments in which diets with a high-Ca, low-P ratio are used and in which P is without doubt the limiting factor as regards calcification. In such experiments the addition of P to the diet improves both Ca and P absorption and bone calcification. In the present experiment, however, where Ca is the limiting factor, additions of P either as phytate or phosphate reduce Ca absorption, as has been shown above, but result in an increased absorption of P. Phosphate, which has the smaller depressing effect on Ca absorption, increases the P absorption to a greater extent than phytate. Thus after 4 weeks, when there are still body reserves of vitamin D, the addition of phytate increases P absorption from 267 mg. (54) to 393 mg. (52) daily, and the addition of phosphate increases it from 267 mg. (54) to 495 mg. (53) daily. Later (13 weeks), when the vitamin reserves are very low, the P absorptions are 191 mg. (54, no extra P), 385 mg. (52, phytate), and 589 mg. (53, phosphate). Most of the additional P absorbed from the high-P diets is excreted via the urine, so that the amounts of P retained by the three animals are approximately the same.

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(4) The addition of either type of P appears to hasten

the exhaustion of body reserves of vitamin D, but phytate P probably acts more quickly than an equal amount of P as phosphate.

(5) Increasing the dietary P by additions of phytate or *phosphate increases P absorption under these conditions,* and even when the vitamin D supply is insufficient, the absorption of P is increased to a greater extent by phosphate than by phytate.

Experiment 14 shows quite clearly that the Ca:P ratio *is not a reliable indication of the calcifying qualities of a* diet. It is true that, when the dietary supply of vitamin D is very low and the body reserves are depleted, lowering the Ca:P ratio by additions of P will reduce Ca absorption. On the other hand, during the early part of the experiment, *when there is a sufficiency of vitamin D, there is a distinct* difference in the results produced by altering the ratio to the same extent by the addition of phytate and phosphate respectively. This difference is not due to unavailable P of the phytate reducing the need for Ca, for puppy 52 (phytate) has absorbed *more* P, some of which must come from the phytate, than 54 (no addition), although the Ca absorption is much reduced. Thus some of the P fed as phytate is available for absorption and it seems probable that it is only the remainder which, combining with Ca in the gut and thereby removing it from the influence of absorptive processes, reduces Ca absorption (Harrison and Mellanby, 1939).

These experiments on dogs demonstrate that the calcifying qualities of a diet can only be judged when a number of factors, including the presence or absence of vitamin D, the total amounts of Ca and P in the diet and the form of the P, are known. The form of the dietary P appears in these experiments to be a crucial factor and it now seems desirable to find out how phytate, as compared with phosphate, decreases Ca absorption and whether, in fact, there

is a quantitative relationship between the excretion of these two substances, so that an increase in phytate excretion brings with it an increase in Ca excretion. In the next experiment, therefore, whilst keeping the total dietary P and Ca equal, three different levels of phytate P are given in order to test the possibility of this relationship.

4. The Effect of Increasing the Phytate, but Not the Total Phosphorus, in Diets with Constant Vitamin D on Phytate and Ca Excretion

EXPERIMENT 15

The object of this experiment was to determine whether or not there was a relationship between the phytate fed, phytate excreted and Ca excreted when the total P, total Ca and vitamin D intakes were constant.

The pre-experimental diet of the puppies contained no cod-liver oil or other source of vitamin D.

Basal experimental diet:

White flour	20–160 g.
Separated milk powder	20 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1% of cereal
Baker's yeast	12% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 265 mg., final 296 mg.)
(P content: initial 209 mg , final 377 mg.)

Daily additions to basal diet:

No of puppy	Mg phytate P per 100 g flour	Mg. phosphate P* per 100 g. flour	Vitamin D, i.u
55 (3107)	300	18	20
56 (3108)	150	168	20
57 (3109)	0	318	20

* Na phytate hydrolysed by phytase of yeast.

Age at beginning of experiment: $6\frac{1}{2}$ weeks.
Duration of experiment: $16\frac{1}{2}$ weeks.

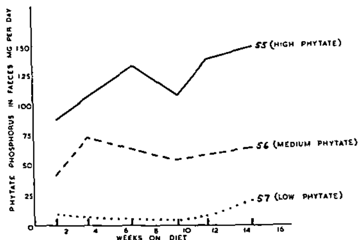
TABLE 27 (Exp. 15)

No of puppy	Dietary conditions				
	Phosphorus added to basal diet (mg per day)				Vitamin D_2, i u
	Na phytate		Na phosphate*		
	Min.	Max	Min	Max.	
55	60	480	4	29	20
56	30	240	34	269	20
57	0	0	64	509	20

* Na phytate hydrolysed by phytase of yeast.

The effect of the different diets tested in this experiment on the phytate and Ca in the faeces is shown in Figs. 58*a* and 58*b*. The amount of dietary phytate varies greatly (high in puppy 55, low in 57 and intermediate in 56), and it is found that the unabsorbed phytate is related to the intake. Although the Ca, P and vitamin D content of the food of these animals is identical, a high phytate-low phosphate intake is accompanied not only by a high phytate but also by a high Ca excretion, a low phytate high phosphate intake by both a low phytate and low Ca excretion, whilst an intermediate phytate intake results in an intermediate Ca excretion. It is evident, therefore, that under the conditions of this experiment the amount of phytate in the diet assumes a position of great importance. Fig. 58*a* leaves no doubt as to the Ca-depriving action of phytate as compared with phosphate of the same P content, when vitamin D is available to the animal.

Comparison of the Ca excretions of puppies 55, 56 and 57 after only two weeks of the experimental period, when the total daily intake of phosphorus is 451 mg., shows that



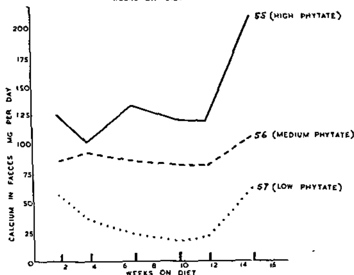


FIG 58 (Exp. 15). Effect of increasing the phytate P, but not the total P of the diet, on the faecal excretion of phytate and Ca.
FIG a. In the presence of vitamin D, increasing the phytate of the diet when the total P intake was kept constant raised the faecal phytate; Fig. b, in the presence of vitamin D increasing the phytate of the diet when the total P intake was kept constant raised the faecal calcium.

an increase in the phytate P from 21 mg. in the diet of puppy 57 to 111 mg. in that of 56 and from 111 mg. in the diet of 56 to 201 mg. in that of 55 produces in both instances about a 50 per cent increase in Ca excretion (Fig. 58*b*).

Only three animals have been described in this experiment, although tests were carried out on six. The group not mentioned above received the same amounts and forms of P and Ca, but the vitamin D intake was 100 i.u. instead of 20 i.u. daily. This increase in vitamin D supplies did not appear to modify the Ca-depriving effect of phytate in this instance. In the next experiment, therefore, the effect of giving 20, 100 and 1000 i.u. respectively of vitamin D daily at two levels of phytate intake will be determined.

5. The Effect of Different Quantities of Dietary Vitamin D_2 on Calcium Absorption

(a) Na phytate and Na phosphate

EXPERIMENT 16

The object of this experiment was to test the effects of large increases in dietary vitamin D on Ca excretion and mineral content of the bones in the presence of high and low dietary phytate.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) From the age of 3 weeks each puppy received 2.5 ml. of cod-liver oil daily for 23 days, after which period the oil was discontinued.

Basal experimental diet:

White flour....	50–160 g.	
Separated milk powder .	30– 20 g.	(reduced after 3 weeks on diet)
Lean meat....	15 g.	
Peanut oil.	11 cc.	

NaCl	... 1% of cereal
Baker's yeast	. 12% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 367 mg., final 293 mg.)
(P content: initial 305 mg., final 372 mg.)

Daily additions to basal diet:

No of puppy	Mg. phosphorus per 100 g. flour: Na phytate	Mg. phosphorus per 100 g. flour: Na phosphate*	Vitamin D_2, i u
58 (3306)	0	318	20
59 (3307)	300	18	20
60 (3310)	0	318	100
61 (3311)	300	18	100
62 (3308)	0	318	1000
63 (3309)	300	18	1000

* Prepared by hydrolysing sodium phytate with yeast phytase.

Age at beginning of experiment: 7 weeks.
Duration of experiment: $15\frac{1}{2}$ weeks.

TABLE 28 (Exp. 16)

Relative effects of phytate and phosphate on bone calcification with different amounts of dietary vitamin D.

No of puppy	Dietary conditions					Bone results		
	Phosphorus added to basal diet (mg per day)				Vitamin D_2, i.u	Combined weight of ash of humerus, radius and ulna, g	Combined weight of ash of 5th, 6th, 7th and 8th ribs, g	A/R ratio of femur shaft
	Na phytate		Na phosphate*					
	Min	Max	Min.	Max.				
58	0	0	159	509	20	6.555	0.987	1.13
59	150	480	9	29	20	5.762	0.678	0.87
60	0	0	159	509	100	6.449	0.958	1.25
61	150	480	9	29	100	5.053	0.724	1.07
62	0	0	159	509	1000	6.732	0.929	1.26
63	150	480	9	29	1000	5.385	0.695	1.19

* Na phytate hydrolysed by phytase of yeast.

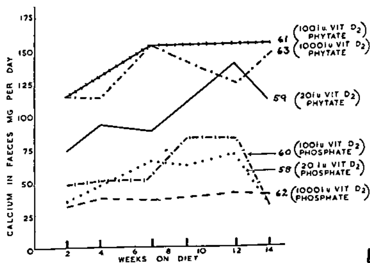


Fig. 59 (Exp. 16). Effects of increasing the dietary vitamin D_2 on the faecal excretion of phytate and calcium.

Note: (1) Increasing the dietary phytate at the expense of phosphate increased both the phytate (Fig. a) and to some extent the Ca (Fig. b) in the faeces;

(2) A large increase of vitamin D_2, i.e. from 20 to 1000 i.u. daily, did not greatly alter the effect of the dietary phytate on phytate (Fig. a) and Ca (Fig. b) excretion.

Figs. 59*a* and 59*b* show that, as in the previous experiment, a high phytate intake is accompanied by both a high phytate and a high Ca excretion. The point to be noted from these figures is that increasing the vitamin D intake

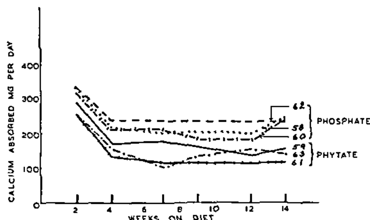


Fig. 60 (Exp 16) Relative effects of phytate and phosphate on calcium absorption in the presence of different amounts of vitamin D_2

No of puppy	Vitamin D_2 (i u)	
58	20	Phosphate
59	20	Phytate
60	100	Phosphate
61	100	Phytate
62	1000	Phosphate
63	1000	Phytate

Note· Increasing the vitamin D_2 intake fifty-fold (from 20 to 1000 i.u. daily) did not appear to influence the effect of phytate in reducing calcium absorption

from 20 i.u. in the case of puppies 58 and 59 to 100 i.u. in 60 and 61, and to 1000 i.u. in 62 and 63, does not greatly increase the amount of Ca absorbed (Fig. 60) and does not significantly alter the depressing effect of phytate on Ca absorption. This result is in marked contrast to the

difference observed in experiment 13 (Fig. 56) between an animal having no vitamin D in the food or body and one having a relatively small amount, 20 i.u. daily. In such a case the presence of vitamin D was the determining factor as regards Ca absorption from the gut. On the other hand, Fig. 59*b* shows that under the conditions of experiment 16, i.e. when once the vitamin D intake reached a certain level, the phytate in the food again assumes a position of much importance and greatly affects the amount of Ca excreted. It will be seen later, in experiment 20, where oatmeal is the source of the dietary phytate, that increasing the vitamin D above 20 i.u. increases Ca absorption under some conditions. Whether this is due to the fact that the phytate is a constituent of the oatmeal and not, as in experiment 16, free Na phytate, or whether it is due to other variations between the two experiments is not known. Clearly 20 i.u. of vitamin D_2 daily are near the 'limiting' amount, i.e. the level of intake which produces maximum absorption of Ca from the gut but may not always be sufficient.

Whilst little or no rickets is produced under the conditions of experiment 16 because of the presence of vitamin D, the effect of phytate as compared with phosphate is to reduce the amount of Ca in the bones. As is shown in Table 28, the average ash weight of the humerus, radius and ulna of the three phytate animals is 5.4 g. and that of the corresponding bones of the phosphate animals 6.579 g. Thus the relative effects of phytate and phosphate on the absorption of Ca from the alimentary canal (as seen in Fig. 60) are reflected in the amount of Ca in the bones. It has also been seen that variations in the daily amount of vitamin D do not cause significant differences in the amount of Ca absorbed from the gut, either in the phytate or in the phosphate animals. In keeping with this result, it will also be noticed that the combined mineral ash of

the humerus, radius and ulna is similar, namely 6.555, 6.449 and 6.732 g. in the phosphate animals and 5.762, 5.053 and 5.385 g. in the three animals having phytate. Thus, the amount of Ca salts deposited in the bones, while greatly affected by phytate as compared with phosphate in the food, is not significantly affected by increasing the amount of dietary vitamin D above a certain quantity.

On the other hand, when the A/R ratios of the femur shafts are considered, it is evident that increasing the *vitamin D causes an improvement in the quality of the* bone formed. This is especially the case in the phytate animals, where the A/R ratios are 0.87 (20 i.u. vitamin D), 1.07 (100 i.u.) and 1.19 (1000 i.u.). In comparison the phosphate animals show a relatively small improvement in these ratios with increasing doses of vitamin D, so that, although increasing the vitamin D in the presence of phytate has no effect on the amount of Ca deposited in the bones, it causes an improvement in the structure of the bones which is indicated by the better A/R ratios. The same relationship is found in the ribs. Plate XXIV (*a*–*c*) shows the radiographic appearance of the costochondral junctions of three animals and it will be seen that, in the presence of 20 i.u. of vitamin D_2, a diet in which the P is mainly phosphate (*a*) produces well formed bones, whereas when the phosphate is largely replaced by phytate of equal P content, the bones are thick and osteoporotic (*b*). Thus, in the presence of equal dietary vitamin D, replacing phosphate by phytate lowers Ca absorption (Fig. 60), reduces the mineral content of the ribs (Table 28) and, as would be expected, reduces the quality of the bones (Plate XXIV, *a* and *b*).

The effect of raising the vitamin D intake from 20 to 1000 i.u. daily with equal dietary phytate is more interesting, for it greatly improves the structure of the bone (cf. Plate XXIV *b* and *c*) without significantly altering the

difference observed in experiment 13 (Fig. 56) between an animal having no vitamin D in the food or body and one having a relatively small amount, 20 i.u. daily. In such a case the presence of vitamin D was the determining factor as regards Ca absorption from the gut. On the other hand, Fig. 59*b* shows that under the conditions of experiment 16, i.e. when once the vitamin D intake reached a certain level, the phytate in the food again assumes a position of much importance and greatly affects the amount of Ca excreted. It will be seen later, in experiment 20, where oatmeal is the source of the dietary phytate, that increasing the vitamin D above 20 i.u. increases Ca absorption under some conditions. Whether this is due to the fact that the phytate is a constituent of the oatmeal and not, as in experiment 16, free Na phytate, or whether it is due to other variations between the two experiments is not known. Clearly 20 i.u. of vitamin D_2 daily are near the 'limiting' amount, i.e. the level of intake which produces maximum absorption of Ca from the gut but may not always be sufficient.

Whilst little or no rickets is produced under the conditions of experiment 16 because of the presence of vitamin D, the effect of phytate as compared with phosphate is to reduce the amount of Ca in the bones. As is shown in Table 28, the average ash weight of the humerus, radius and ulna of the three phytate animals is 5.4 g. and that of the corresponding bones of the phosphate animals 6.579 g. Thus the relative effects of phytate and phosphate on the absorption of Ca from the alimentary canal (as seen in Fig. 60) are reflected in the amount of Ca in the bones. It has also been seen that variations in the daily amount of vitamin D do not cause significant differences in the amount of Ca absorbed from the gut, either in the phytate or in the phosphate animals. In keeping with this result, it will also be noticed that the combined mineral ash of

the humerus, radius and ulna is similar, namely 6.555, 6.449 and 6.732 g. in the phosphate animals and 5.762, 5.053 and 5.385 g. in the three animals having phytate. Thus, the amount of Ca salts deposited in the bones, while greatly affected by phytate as compared with phosphate in the food, is not significantly affected by increasing the amount of dietary vitamin D above a certain quantity.

On the other hand, when the A/R ratios of the femur shafts are considered, it is evident that increasing the vitamin D causes an improvement in the quality of the bone formed. This is especially the case in the phytate animals, where the A/R ratios are 0.87 (20 i.u. vitamin D), 1.07 (100 i.u.) and 1.19 (1000 i.u.). In comparison the phosphate animals show a relatively small improvement in these ratios with increasing doses of vitamin D, so that, although increasing the vitamin D in the presence of phytate has no effect on the amount of Ca deposited in the bones, it causes an improvement in the structure of the bones which is indicated by the better A/R ratios. The same relationship is found in the ribs. Plate XXIV (*a*–*c*) shows the radiographic appearance of the costochondral junctions of three animals and it will be seen that, in the presence of 20 i.u. of vitamin D_2, a diet in which the P is mainly phosphate (*a*) produces well formed bones, whereas when the phosphate is largely replaced by phytate of equal P content, the bones are thick and osteoporotic (*b*). Thus, in the presence of equal dietary vitamin D, replacing phosphate by phytate lowers Ca absorption (Fig. 60), reduces the mineral content of the ribs (Table 28) and, as would be expected, reduces the quality of the bones (Plate XXIV, *a* and *b*).

The effect of raising the vitamin D intake from 20 to 1000 i.u. daily with equal dietary phytate is more interesting, for it greatly improves the structure of the bone (cf. Plate XXIV *b* and *c*) without significantly altering the

Ca absorption (Fig. 60) or the mineral ash of the bone (Table 28). The *thin compact bones of puppy 63 (phytate, 1000 i.u. vitamin D)* are comparable in radiographic appearance to those of puppy 58 (phosphate, 20 i.u. vitamin D).



PLATE XXIV

Experiment 16. a to c. Radiographs of costochondral junction.
a Puppy 58. Received phosphate with 20 i.u. vitamin D_2.
b Puppy 59. Received phytate with 20 i.u. vitamin D_2.
c Puppy 63. Received phytate with 1000 i.u. vitamin D_2.
Note: (1) substituting phytate P (b) for equal amounts of inorganic P (a) resulted in thicker and more osteoporotic bones and reduced mineral ash content (see Table 28);
(2) increasing vitamin D_2 from 20 (b) to 1000 i.u. (c) reduced the bulk and improved the quality of the bone although the mineral ash content was not increased (see page 321 and discussion page 346).

It is obvious, therefore, that, when phytate forms a substantial part of the diet, *raising the vitamin D intake,* although not ensuring a higher Ca absorption or higher mineral content of the bones, will produce better calcified and more compact bones.

Note: In the course of this work it became clear that the A/R ratio (Chick, Korenchevsky and Roscoe, 1926; Chick and Roscoe, 1926), when A is the weight of the mineral ash of the bone and R

the weight of the dried, fat-extracted bone less the weight of the ash, has only a limited usefulness as a measure of Ca retention.

Obviously this ratio may be varied by alterations in the weight of mineral ash when the organic tissue is constant or, in the case of constant amounts of mineral ash, *by alterations in the amount* of organic tissue. When, therefore, it became apparent that alterations in vitamin D supplies might vary the A/R ratio and not the ash content (cf. puppies 59 and 63, Table 28), the ratio as a measure of calcification was abandoned.

In experiment 15 the close relationship between phytate and Ca in the faeces was seen, a rise or fall in the excretion of phytate being associated with a similar change in the excretion of Ca. Experiment 16 showed that large increases in vitamin D could not, under the conditions of that experiment, overcome the depressing effect of phytate on Ca absorption. It seemed likely that these unabsorbed substances were associated in the faeces, probably as an insoluble Ca phytate, and that when this combination took place in the intestine the Ca became unavailable to the animal. It was therefore of interest to determine whether *the animal could make use of Ca phytate if the substance* were presented in the food in an insoluble form and, if so, how the availability of this substance compared with that of Ca phosphate. It was probable that there would be some breakdown of each in the stomach, with the formation of soluble Ca chloride. On the other hand, there was an equal possibility that in the duodenum the Ca might react with the phytate and return to its less soluble form. In the following experiment practically all the Ca of the diet was given in the form of either penta-calcium phytate or Ca phosphate, but only about 70% of the total P intake came from the Ca phytate. The object was to see to what extent dogs could make use of the Ca and P of these compounds and whether a large increase of vitamin D in the diet facilitated their absorption.

(b) Ca phytate or Ca phosphate as source of dietary Ca

EXPERIMENT 17

The objects of this experiment were (1) to test the effect of large increases in dietary vitamin D on the availability of the Ca of Ca phytate, and (2) to determine the relative availability of Ca fed as Ca phytate and Ca phosphate respectively when there is plenty of vitamin D available.

The pre-experimental diet of the puppies contained no cod-liver oil for the 3½ weeks prior to the beginning of the experimental period.

Basal experimental diet:

White flour	80–120 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 22 mg., final 30 mg.)
(P content: initial 125 mg., final 175 mg.)
Note: No separated milk powder; hence Ca of diet low.

Daily additions to basal diet:

	Penta-Ca phytate*	Ca phosphate	Vitamin D_2, i.u.
†64 (3313)	+	–	0
65 (3314)	+	–	20
66 (3315)	+	–	1000
67 (3316)	–	+	1000

* Each batch was tested independently, the Ca:P ratios varying from 1.03 to 1.1.
† See note under Table 9.

Age at beginning of experiment: About 8 weeks.
Duration of experiment: 9 weeks.

TABLE 29 (Exp. 17)

Relative availability of the calcium of calcium phytate and calcium phosphate

No. of puppy	Dietary Conditions									Bone results		
	Additions to basal diet									Combined weight of ash, humerus, radius and ulna	A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.†
	Calcium				Phosphorus				Vitamin D_2, i.u.			
	Total*		Phytate		Total*		Phytate					
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.				
‡64	212	366	191	322	319	469	200	300	0	3.077	0.96	5
65	272	366	256	322	408	469	250	300	20	4.656	1.10	0
66	272	366	256	322	408	469	250	300	1000	4.586	1.11	0
67	308	366	0	0	432	469	44	55	1000	5.129	1.31	0

* These figures were obtained from estimations on the cooked food. The Ca as phytate figures were calculated.

† Rickets graded 1 to 10, the number increasing with the severity of the disease.

‡ As this animal did not eat well, 200 i.u. of vitamin D_2 were injected intravenously on the 7th day. The appetite improved but it was not possible to give it the same amount of cereal and Ca phytate as the rest of the family until towards the end of the experiment.

It is seen from Fig. 61 that puppies 65 and 66, both having Ca phytate and vitamin D_2 in addition to the basal diet, absorb sufficient Ca to maintain a positive balance of between 100 and 200 mg. daily throughout the experimental period, but that 66, receiving 1000 i.u. of the vitamin daily, absorbs no more Ca than 65, which has only 20 i.u. Both, on the other hand, absorb much less than puppy 67, which has the larger dosage of vitamin D_2 and the same amount of calcium as 65 and 66, but as phosphate instead of phytate. The calcium absorbed by the phytate animals during the experimental period can only have

come from the Ca phytate in the food. The third of these, puppy 64, which has no vitamin D in its diet, loses its ability to absorb Ca after 6½ weeks of the experiment, in spite of the fact that it does not eat as much as the others, grows less, and at an early stage of the experiment had one dose of 200 i.u. of vitamin D_2 injected into the blood stream

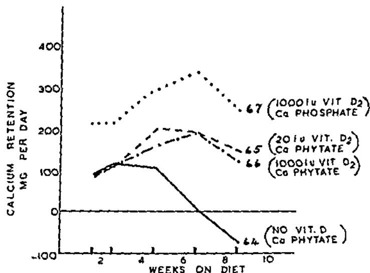


FIG. 61 (Exp 17). Relative effects of Ca phytate and Ca phosphate on Ca retention in the presence of different amounts of dietary vitamin D_2

About 85% of the Ca in the diet was given as calcium phytate in 64, 65, 66 and as calcium phosphate in 67.

Note: In the absence of dietary vitamin D (64) the power to absorb Ca from Ca phytate was soon reduced. 20 i.u. of vitamin D_2 daily (65) promoted a higher retention of Ca, but 1000 i.u. (66) effected no further improvement. With equal amounts of vitamin D_2 (1000 i u), Ca phosphate (67) allowed a much higher Ca retention than did Ca phytate (66).

(see note, p. 329), which no doubt has lengthened the period during which absorption takes place. It is therefore probable that in the complete absence of vitamin D the Ca of Ca phytate is unavailable.

When bone calcification is considered it will be seen that

the mineral ash weights (Table 29), as would be expected from the Ca absorption, are low in 64, high in 67 and equal at an intermediate level in 65 and 66. The A/R ratios show that 67 (phosphate) has better calcified bone than the three phytate animals, but the differences between 64 (D-deficient), 65 (20 i.u. vitamin D) and 66 (1000 i.u.) are small. This may be due partly to the short experimental period and relatively poor bone growth and partly to the injection of 200 i.u. of vitamin D_2 given to 64.

Thus the results of this experiment are in keeping with the findings of experiments 14 and 15, namely that phytate in the presence of vitamin D can reduce both the Ca absorption and the amount of Ca deposited in the bones to a greater extent than phosphate of equal P content, and further that a large increase in dietary vitamin D does not offset this effect. It also shows that an animal supplied with vitamin D can not only make use of Ca given as an insoluble penta-calcium phytate but can in some way deal with the phytate and reduce the amount excreted. Thus when the diet contains vitamin D (20 or 1000 i.u. daily) only about 40 to 60 per cent of the P fed as phytate is recovered from the faeces.

All the foregoing experiments have shown that phytate, even in the presence of abundant vitamin D, reduces Ca absorption when compared with phosphate and that this results in less well calcified bones. The practical problem is how to overcome this effect and without doubt the simplest method is to increase the Ca content of the diet.

It was shown in 1925 (E. Mellanby) that the addition of Ca to a low-Ca, vitamin D-deficient diet consisting largely of cereals resulted in improved calcification, with a delay in the onset of rickets. It is now proposed to demonstrate that this was probably largely due to the fact that the body contained reserves of vitamin D. Additional dietary Ca protects against rickets in the early part of the

experiment while there are still reserves of the vitamin in the body, but as these disappear much of the additional Ca is excreted and rickets rapidly develops.

(c) *The interaction of additional calcium and phytate*

EXPERIMENT 18

Object. As experience has shown that the excretion of calcium in the faeces increases with the exhaustion of the vitamin D reserves of the body (p. 302), this experiment was made with the object of determining whether such increase could be compensated for by gradually raising the calcium intake as the vitamin D stores were depleted.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) From the age of 4 weeks each puppy was given 2.5 ml. daily of cod-liver oil until the experiment was begun, so that all would have had some body stores of vitamin D.

Basal experimental diet:

Cereal (oatmeal)	45–90 g.
Separated milk powder	10–20 g.
Lean meat	15 g.
Peanut oil	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A naphthoate	1000 i.u.

(Ca content initial 170 mg., final 347 mg.)
(P content: initial 308 mg., final 511 mg.)

Daily additions to basal diet:

68 (2889)	0– 46 mg. calcium as $CaCO_3$
69 (2890)	136– 300 mg. calcium as $CaCO_3$
70 (2891)	540–1070 mg. calcium as $CaCO_3$

Age at beginning of experiment: 8 weeks.
Duration of experiment: 15½ weeks.

TABLE 30 (Exp. 18)

The effect of increasing the dietary calcium, as the vitamin D reserves of the body are depleted, on bone calcification

No of puppy	Mg Ca added to basal diet	Ca:P ratio of diet		Weight of mineral ash of femur shaft	A/R ratio of femur shaft	Grade of rickets as judged by X-rays* after·		
		Initial	Final			$7\frac{1}{2}$ wks. on diet	$10\frac{1}{2}$ wks. on diet	$15\frac{1}{2}$ wks on diet
68	0– 46	0.56 :1	0.77:1	0.64	1.01	3	5	8
69	136– 300	0.995:1	1.25:1	1.28	1.05	1	2	7
70	540–1070	1.98 :1	2.77:1	1.18	1.02	1	2	7

* Rickets graded from 1 to 10, the number increasing with the severity of the disease.

Fig. 62 shows that, although in the early stages of the experiment, when bodily reserves of vitamin D are present, *much of the extra calcium is absorbed, later even 1400 mg.* of calcium, and a dietary Ca:P ratio of 2.77:1, is ineffective in maintaining a positive calcium absorption. It is probable that puppy 70 has excreted, via the urine, some at least of the extra Ca absorbed in the early part of the experiment but unfortunately urine samples from these animals were not collected. After $7\frac{1}{2}$ weeks there is obvious rickets in puppy 68, whereas at this time 69 and 70 are nearly normal, but three weeks later the latter two are becoming definitely rachitic, 69 being rather worse that 70. By the end of the experiment all three have severe rickets; the condition of puppy 68 is the worst but that of 69 and 70 is only slightly better and it is difficult to differentiate between them by X-rays. It appears from Fig. 62 that the additional calcium in the diet is effective in bringing about increased calcium retention but only as long as the reserves of vitamin D are present, and that when the reserves are used up most of the Ca of the diet is excreted.

Some of the additional Ca given to puppies 69 and 70 has evidently been deposited in the bones, for the ash

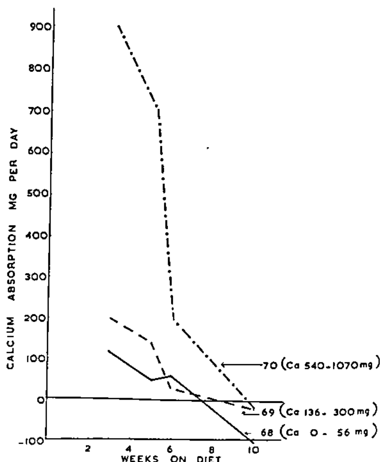


Fig. 62 (Exp. 18). The effect of increasing the dietary calcium on calcium absorption as the vitamin D reserves of the body are depleted.

Note: The loss of power to absorb Ca, even on a high dietary Ca intake (puppy 70), after 5 weeks on a diet devoid of vitamin D.

weights of their femur shafts are about double those of puppy 68. The A/R ratios in these two cases, however, show no improvement, indicating that, when there is no

dietary vitamin D, increasing the mineral content of a bone may not improve its quality. This is the converse of what was found in experiment 16, where it was shown that increasing vitamin D supplies might improve bone quality without altering the mineral content.

EXPERIMENT 19

Object. This experiment was made to test (1) the effect on bone calcification and on calcium absorption of adding Ca to a basal diet rich in phytate—in this case sodium phytate—and (2) the effect of the additional Ca on the excretion of phytate P in the presence and in the absence of dietary vitamin D.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) For about 2 weeks before the experiment was begun each of the puppies had 2.5 ml. daily of cod-liver oil, so that all would have had some bodily stores of vitamin D.

Basal experimental diet:

Bread ..	. 64–150 g.
Separated milk powder	20 g.
Lean meat.. .	7.5 – 17 5 g.
Peanut oil..	5.6 – 13 ml.
NaCl	0 75– 1.75 g.
Baker's yeast	2.25– 5.25 g
Ascorbic acid.	5 mg.
Cabbage .	10 5 – 24 5 g.

(Ca content of above initial 290 mg., final 398 mg.)
(P content of above. initial 332 mg , final 500 mg.)

Daily additions to basal diet:

No of puppy	Phosphorus as Na phytate, mg	Calcium (as $CaCO_3$), mg	Vitamin D_2, I U.
71 (2S64)	200	None	None
72 (2S67)	200	400	None
73 (2S65)	200	None	1000
74 (2S66)	200	400	1000

Age at beginning of experiment: $6\frac{1}{2}$ weeks.
Duration of experiment: 15–16 weeks.

TABLE 31 (Exp. 19)

Effect of additional dietary calcium on bone calcification in the presence and absence of dietary vitamin D

No. of puppy	Dietary conditions		Bone results	
	Daily additions to basal diet			
	Ca as $CaCO_3$, mg.	Vitamin D_2, i u.	A/R ratio of femur shaft	Rickets* as judged by X-rays at P.M.
71 (2864)	0	0	0.43	10
72 (2867)	400	0	1.19	2
73 (2865)	0	1000	1.24	0
74 (2866)	400	1000	1.53	0

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

In this experiment 200 mg. of phytate P were included in the basal diet of all puppies.

Fig. 63 shows that, in the presence of abundant dietary vitamin D_2, increasing the calcium intake leads to a greatly increased calcium absorption, especially in the earlier days of the feeding period (see 74 as compared with 73). When there is no vitamin D in the diet, but the reserves are moderately good, a high calcium intake, as in the case of puppy 72, also causes a large calcium absorption. As the reserves are used up, however, most of the additional dietary Ca is excreted and the amount absorbed diminishes, so that by the 7th week of the experiment only about 50 mg. daily are absorbed as compared with 450 mg. in the early days of the feeding period. After this interval there is little or no difference in the amount of calcium absorbed by puppies 71 and 72, receiving the lower and higher calcium diets respectively.

The effect of the large absorption of calcium by puppy 72 during the early weeks on the high Ca diet is greatly to delay the onset of rickets. After 11½ weeks of the experiment the radiographic appearance of this animal is nearly normal, whereas puppy 71, with no extra Ca, has developed very severe rickets, and even at the end of the feeding period there is much less rickets in 72 than 71.

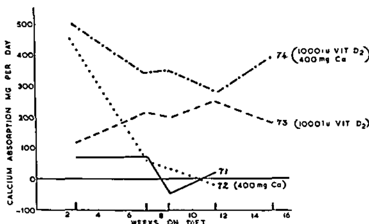


FIG. 63 (Exp. 19). Effect of different dietary calcium intakes on calcium absorption in the presence and absence of vitamin D_2.
Note: Ca absorption was highest throughout in 73 and 74 (1000 i.u. vitamin D_2) but was specially high in 74 (extra 400 mg. Ca). After 7 weeks of the D-deficient diet (71 and 72) the power to absorb Ca was much reduced even in the presence of an extra 400 mg. of Ca (72). 200 mg. P as sodium phytate were included in the daily diets of all.

In view of the close relationship between phytate P and Ca excretion demonstrated in experiments 16, 17 and 19, it would be expected that the greatly increased excretion of Ca by puppy 72 following the loss of its vitamin D reserves would result in an increased excretion of phytate. Fig. 64 shows that this is not so, for in the absence of vitamin D from the diet the additional Ca has but little effect on the phytate excretion. Puppy 72 (high Ca) even

Age at beginning of experiment: 6½ weeks.
Duration of experiment: 15–16 weeks.

TABLE 31 (Exp. 19)

Effect of additional dietary calcium on bone calcification in the presence and absence of dietary vitamin D

No. of puppy	Dietary conditions: Daily additions to basal diet		Bone results	
	Ca as $CaCO_3$, mg.	Vitamin D_2, i.u.	A/R ratio of femur shaft	Rickets* as judged by X-rays at P.M.
71 (2884)	0	0	0.65	10
72 (2887)	400	0	1.19	2
73 (2885)	0	100	1.24	0
74 (2886)	400	100	1.53	0

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

In this experiment 200 mg. of phytate P were included in the basal diet of all puppies.

Fig. 63 shows that, in the presence of abundant dietary vitamin D_2, increasing the calcium intake leads to a greatly increased calcium absorption, especially in the earlier days of the feeding period (see 74 as compared with 73). When there is no vitamin D in the diet, but the reserves are moderately good, a high calcium intake, as in the case of puppy 72, also causes a large calcium absorption. As the reserves are used up, however, most of the additional dietary Ca is excreted and the amount absorbed diminishes, so that by the 7th week of the experiment only about 50 mg. daily are absorbed as compared with 450 mg. in the early days of the feeding period. After this interval there is little or no difference in the amount of calcium absorbed by puppies 71 and 72, receiving the lower and higher calcium diets respectively.

The effect of the large absorption of calcium by puppy 72 during the early weeks on the high Ca diet is greatly to delay the onset of rickets. After 11½ weeks of the experiment the radiographic appearance of this animal is nearly normal, whereas puppy 71, with no extra Ca, has developed very severe rickets, and even at the end of the feeding period there is much less rickets in 72 than 71.

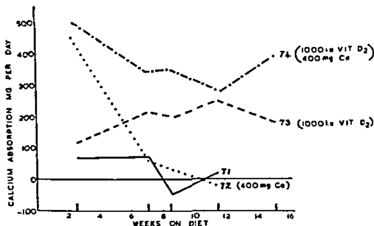


FIG 63 (Exp. 19). Effect of different dietary calcium intakes on calcium absorption in the presence and absence of vitamin D_2. *Note:* Ca absorption was highest throughout in 73 and 74 (1000 i.u vitamin D_2) but was specially high in 74 (extra 400 mg. Ca). After 7 weeks of the D-deficient diet (71 and 72) the power to absorb Ca was much reduced even in the presence of an extra 400 mg. of Ca (72). 200 mg. P as sodium phytate were included in the daily diets of all.

In view of the close relationship between phytate P and Ca excretion demonstrated in experiments 16, 17 and 19, it would be expected that the greatly increased excretion of Ca by puppy 72 following the loss of its vitamin D reserves would result in an increased excretion of phytate. Fig. 64 shows that this is not so, for in the absence of vitamin D from the diet the additional Ca has but little effect on the phytate excretion. Puppy 72 (high Ca) even

Age at beginning of experiment: $6\frac{1}{2}$ weeks.
Duration of experiment: 15–16 weeks.

TABLE 31 (Exp. 19)

Effect of additional dietary calcium on bone calcification in the presence and absence of dietary vitamin D

No. of puppy	Dietary conditions		Bone results	
	Daily additions to basal diet		A/R ratio of femur shaft	Rickets* as judged by X-rays at P.M.
	Ca as $CaCO_3$, mg	Vitamin D_2, I.U.		
71 (2864)	0	0	0.43	10
72 (2867)	400	0	1.19	2
73 (2865)	0	1000	1.24	0
74 (2866)	400	1000	1.53	0

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

In this experiment 200 mg. of phytate P were included in the basal diet of all puppies.

Fig. 63 shows that, in the presence of abundant dietary vitamin D_2, increasing the calcium intake leads to a greatly increased calcium absorption, especially in the earlier days of the feeding period (see 74 as compared with 73). When there is no vitamin D in the diet, but the reserves are moderately good, a high calcium intake, as in the case of puppy 72, also causes a large calcium absorption. As the reserves are used up, however, most of the additional dietary Ca is excreted and the amount absorbed diminishes, so that by the 7th week of the experiment only about 50 mg. daily are absorbed as compared with 450 mg. in the early days of the feeding period. After this interval there is little or no difference in the amount of calcium absorbed by puppies 71 and 72, receiving the lower and higher calcium diets respectively.

the additional Ca to increase phytate excretion when the diet is deficient in vitamin D.

It is evident from this experiment that, even when there is no vitamin D in the diet, the body reserves of this substance can be very effective in promoting the absorption of any additional calcium there is in the food, but may have less effect on phytate excretion. The presence of dietary vitamin D promotes the absorption of some of the additional Ca, but that not absorbed appears to increase the excretion of phytate.

EXPERIMENT 20

The object of the present experiment was to test the effect of adding calcium to diets containing smaller amounts of vitamin D_2 than were given in experiment 19, namely 0, 5, 20 and 100 i.u. respectively, as it was thought likely that the effect of raising the Ca intake might vary with the magnitude of the vitamin dosage.

Pre-experimental diet. The litter, when fed independently of the mother, did not receive any additional source of vitamin D.

Basal experimental diet:

Oatmeal	15–175 g.
Separated milk powder. .	25 g.
Lean meat.	15 g.
Peanut oil	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 340 mg., final 443 mg.)
(P content initial 326 mg., final 945 mg.)
(Phytate P content· initial 38 mg., final 438 mg.)

Age at beginning of experiment: 6 weeks.
Duration of experiment: 17 weeks.

after $11\frac{1}{2}$ weeks on diet excretes only about 38 mg. of phytate more than puppy 71 (low Ca). In the presence of dietary vitamin D_2 the addition of dietary Ca definitely causes an increase in phytate excretion, the greatest variation between the puppy having additional Ca (74) and

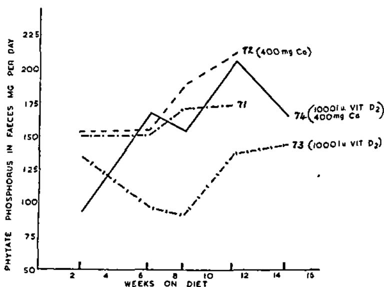


FIG. 64 (Exp. 19). Effect of additional dietary Ca on phytate excretion in the presence and absence of dietary vitamin D_2.

In the presence of vitamin D_2 (1000 i.u.), the addition of 400 mg. of Ca daily (74) increased the faecal phytate (cf. 73). In the absence of the vitamin, the additional 400 mg. Ca (72) did not materially increase the phytate excretion, since much of the phytate ingested was being excreted without the additional Ca (71).

that not receiving it (73) being 77 mg. after $11\frac{1}{2}$ weeks on diet. It will be seen that when no additional Ca is given, 40–60 per cent of the dietary phytate disappears from the gut of the animal receiving vitamin D (73) whereas only 25–35 per cent disappears in the case of the D-deficient animal (71). There is less margin, therefore, for

the additional Ca to increase phytate excretion when the diet is deficient in vitamin D.

It is evident from this experiment that, even when there is no vitamin D in the diet, the body reserves of this substance can be very effective in promoting the absorption of any additional calcium there is in the food, but may have less effect on phytate excretion. The presence of dietary vitamin D promotes the absorption of some of the additional Ca, but that not absorbed appears to increase the excretion of phytate.

EXPERIMENT 20

The object of the present experiment was to test the effect of adding calcium to diets containing smaller amounts of vitamin D_2 than were given in experiment 19, namely 0, 5, 20 and 100 i.u. respectively, as it was thought likely that the effect of raising the Ca intake might vary with the magnitude of the vitamin dosage.

Pre-experimental diet. The litter, when fed independently of the mother, did not receive any additional source of vitamin D.

Basal experimental diet:

Oatmeal..	15–175 g.
Separated milk powder.	25 g.
Lean meat.	15 g.
Peanut oil.	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid.	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 340 mg., final 443 mg.)
(P content: initial 326 mg., final 945 mg.)
(Phytate P content: initial 38 mg., final 438 mg.)

Age at beginning of experiment: 6 weeks.
Duration of experiment: 17 weeks.

TABLE 32 (Exp. 20)

Effect of additional dietary Ca on bone calcification in the presence of different amounts of vitamin D_2

No. of puppy	Dietary conditions		Bone results	
	Daily additions to basal diet		A/R ratio of femur shaft	Rickets as judged by X-rays at P.M.*
	Calcium as $CaCO_3$, mg.	Vitamin D_2, i.u.		
75 (3003)	0	0	0.85	9
76 (3007)	200	0	0.88	8
77 (3004)	0	5	1.04	3
78 (3008)	200	5	1.30	<1
79 (3005)	0	20	1.11	1
80 (3009)	200	20	1.35	0
81 (3006)	0	100	1.27	0
82 (3010)	200	100	1.36	0

* Rickets graded 1 to 10, the number increasing with the severity of the disease.

It will be seen from Table 32 and Fig. 65 that:

(1) Raising the Ca intake by the addition of $CaCO_3$ increases the amount of Ca absorbed, but in the absence of vitamin D the increase is small.

(2) The effect of added Ca varies with the amount of vitamin D given. Thus a dose of 5 i.u. of vitamin D_2 with additional Ca (78) results in approximately the same Ca absorption as 100 i.u. without the additional Ca (81).

(3) Increasing the vitamin D intake from 0 to 100 i.u. when the Ca of the food is low, increases the A/R ratios of the bones (0.85, 1.04, 1.11 and 1.27), but with the food of higher Ca content the improvement in calcification caused by the change of vitamin D intake is negligible except when the increase is from none to 5 i.u. daily.

This experiment indicates, therefore, that the addition of $CaCO_3$ to the diet promotes the absorption of Ca, even

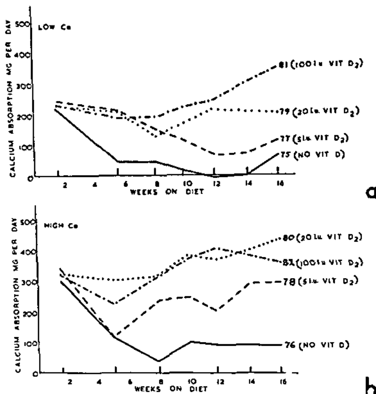


FIG. 65 (Exp. 20). Effect of additional dietary calcium on calcium absorption in the presence and absence of vitamin D_2.

FIG. a, with low Ca intake, increasing vitamin D causes increased calcium absorption; Fig. b, with a higher Ca intake, increasing vitamin D increases the absorption of Ca, except that 80 (20 I.U.) and 82 (100 I.U.) are practically the same (cf. phytate excretion Fig. 66b).

Note: Comparison of Figures a and b shows (1) that at all levels extra dietary Ca has caused increased absorption; (2) that 20 i.u of vitamin D_2 with an additional 200 mg. of Ca (80) had a better effect on Ca absorption than 100 i.u. without extra Ca (81). Similarly 5 I.U. vitamin D_2 with 200 mg. extra Ca (78) brought about better Ca absorption than 20 i.u. vitamin D without extra Ca (79).

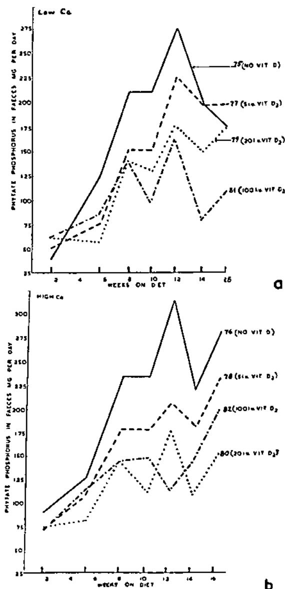


FIG 66 (Exp. 20). Effect of additional dietary calcium on phytate excretion in the presence and absence of dietary vitamin D.
Fig. a, with low Ca intake, increasing vitamin D_2 causes a reduced excretion of phytate.

in the absence of dietary vitamin D, and that the increase in Ca content is of greater significance to the animal's economy when the dietary vitamin D is small.

As regards bone calcification, animals 75 and 76 show that an increased Ca absorption in the absence of vitamin D does not necessarily indicate improved bone quality. Thus puppy 76 (Fig. 65) consistently absorbs more Ca than 75. The mineral ash content of the femur shaft is 23 per cent greater than that of 75, but the A/R ratios (0.88 and 0.85) do not differ significantly. This result is similar to that described on p. 334, and the converse of that given on p. 321.

Turning now to phytate excretion, Fig. 66 shows that with the lower Ca intake the phytate excretion is reduced as the dietary supply of vitamin D improves. In the presence of the additional Ca (Fig. 66*b*) diets containing 5 and 20 i.u. promote progressively lower phytate excretions but increases beyond 20 i.u. have apparently no further effect. Comparing the effect of the additional Ca in animals with otherwise identical diets, it will be seen that in the absence of the vitamin the excretion of phytate is slightly increased, but as the dosage of the vitamin is raised from 5 to 100 i.u. daily the slight effect of the additional Ca disappears.

The reduced effect of the additional Ca on phytate excretion when there is a supply of vitamin D may have been due to the greatly increased Ca absorption reducing the amount of phytate precipitated by this substance in the gut.

Fig. b, with a higher Ca intake, increasing vitamin D reduces phytate excretion except that 80 (20 i.u.) and 82 (100 i.u.) are practically the same.

Note: Comparison of Figures a and b show that (1) in the absence of vitamin D, additional Ca raised the faecal phytate; (2) when vitamin D was given in doses of 5, 20 and 100 i.u. respectively the effect of additional Ca on phytate excretion was small or absent.

It is possible that the addition of a very large amount of calcium to a diet rich in phytate would result in a greater increase in unabsorbed phytate than that observed in these experiments. Such a diet would compare with those of high Ca, low-P content often used by other workers in rat experiments on rickets. On the other hand, extra dietary Ca increases Ca absorption, but only in the presence of vitamin D, either in the diet or as body reserves, can good absorption be maintained and good calcification of bone ensured.

Discussion

Experiments have been described above which formed a part of a further study of the anticalcifying effect of diets containing phytate as compared with those in which the phytate has been replaced by phosphate containing an equal amount of phosphorus.

When it was first established that phytate was the substance in oatmeal and other cereals mainly responsible for their anticalcifying actions, it was thought to be of but little practical importance so long as there was plenty of vitamin D in the food. The results described above showed that this was not the case. In the presence of vitamin D either in the food or when stored in the body, phytate consistently reduced Ca absorption to a greater extent than an equal amount of P given as phosphate. It is true that vitamin D, above a certain minimal level, prevented the development of rickets and of osteomalacia when the diet was rich in phytate and low in Ca, but even when the vitamin D intake was as high as 1000 i.u. daily, phytate still retained the power of reducing the absorption of calcium, thereby tending to produce a more subnormal condition of bone calcification than would be produced by a similar diet containing the same amount of phosphorus but as phosphate.

When it became apparent that the presence or absence of vitamin D was a crucial condition of the problem under study, one of the methods used in the foregoing experiments was to give the puppies a diet which caused the gradual exhaustion of their body stores of the vitamin. A great reduction in the power to absorb Ca was regarded as coincident with the exhaustion of these reserves. The period necessary to produce this condition varied greatly according to the quantity of the vitamin consumed and stored before the experiment began. In some cases it took two or three weeks only, whereas in others it might take two or more months on a vitamin-deficient diet before the growing puppy lost its power to retain Ca. In adult dogs where the demand is probably less and the stores are greater it may take a year before the body is deprived of all traces of the vitamin. In the early stages of the experimental feeding period before vitamin D depletion, phytate significantly lowered Ca absorption when compared with phosphate but later this differentiation disappeared and the absorption was reduced in both cases, leading, if the experiment continued long enough, to negative balances of this element. It was not the effect of phytate in depressing Ca absorption which was fundamentally altered by the loss of the vitamin so much as that inorganic phosphate changed from being relatively without effect on Ca absorption to having an inhibitory action of the same order as phytate.

In the foregoing experimental work two methods of assessing Ca metabolism have been used (i) the determination of Ca and P absorbed and excreted under controlled dietary conditions and (ii) estimation of the degree *of calcification of the bones. Although it is generally true* that more or less Ca absorbed from the intestine means better or worse calcified bones, the two measurements are not identical in their interpretation. Increasing the

vitamin D supply of the body from nil upwards improves Ca absorption greatly once the stores of vitamin D are depleted but soon reaches a point beyond which further increases of vitamin D are ineffective in this respect. It was found, for instance, that 20 i.u. daily of vitamin D_3, which will be referred to as the 'limiting amount' were often just sufficient with the experimental diets used to promote the maximum amount of Ca absorption, and that raising the dosage from 20 to 1000 i.u. in diets in which all other factors were constant did not materially increase the Ca absorption or retention.

Turning now to the question of the structure and calcification of bone, it would be expected that below the limiting amount of vitamin D, bone calcification would be poor and that rickets would be produced, since the amount of Ca absorbed was subnormal. Above this limiting intake, however, since the amount of Ca absorbed remained a maximum under the experimental conditions, it would be expected that the Ca content of comparable bones would also be a maximum i.e. as much in amount in animals with low as in animals with higher vitamin D intake. This indeed, proved to be the case, but it did not mean that bones of animals receiving the larger amount of vitamin D were of the same structure, even though the Ca content was similar. In such cases the radiographic appearance and the A/R ratio of comparable bones sometimes showed that the amount of vitamin D which just promoted a maximum absorption of Ca was not sufficient to produce well calcified bones.

This fact can be seen in the radiographs (plate XXIV, p. 326) of the costochondral junctions of three animals, two of which had received a high phytate diet together with 20 and 1000 i.u. respectively of vitamin D. It will be seen that in puppy 59 (*b*) the ribs are thick and

osteoporotic, whereas in puppy 63 (c) with the higher vitamin D intake, they are relatively well calcified and well formed, yet the bones of these two animals contained approximately the same amount of mineral matter (Table 28). The A/R ratios of the femur shafts of these two animals were in line with the radiographic appearance of the ribs, being lower in the animal with the lower vitamin D intake.

Since the A/R ratio represents the proportion of the total mineral content of the bone to the dried fat-free organic matter, the bone with the lower A/R ratio, but with the same total amount of mineral matter, must have had a larger amount of non-calcified or poorly calcified organic matter, a fact confirmed by radiological and histological examination. The bones of puppy 59 were more cancellous, i.e. they contained more marrow spaces and were also of a more elementary lamellar type of structure with fewer well formed Haversian systems than those of puppy 63 (1000 i.u. vitamin D_2). Of course with a vitamin D intake below the limiting amount, under the experimental conditions chosen, the Ca absorption from the gut was depressed and frank rickets with a large amount of osteoid tissue was produced. In this case the A/R ratio diminished, both because the mineral content was low and because non-calcified tissue, including osteoid tissue, was relatively increased.

Here then is direct evidence that vitamin D not only has the function of controlling Ca absorption and of incorporating it into bone, but above the limiting amount controls the quality and structure of bone laid down, i.e. ensures that not only the pattern of the bone is correct but also that what is formed is more fully calcified. The amount of vitamin D necessary to produce perfect bones is therefore under the experimental conditions studied

higher than the amount necessary to produce maximum Ca absorption from the gut and maximum Ca incorporation in the bone.

Effect of phytate

The effect of increasing dietary phytate in the presence of vitamin D is quite another matter. In numerous experiments described above it has been shown that substituting sodium phytate for phosphate, so as to retain the same total P in the diet, has two constant effects. In the first place it increases the phytate in the faeces and the more consumed the greater is the amount excreted in this way, (fig. 58*a*). It is true that a varying proportion of the phytate eaten disappears, i.e. does not appear in the faeces or urine, and that the amount which disappears is greater when the diet contains vitamin D than when the vitamin is absent from the body. But this action of vitamin D in bringing about the 'disappearance' of phytate from the gut is limited so that, even when the vitamin D intake is raised to a high level other conditions being constant, there is no further diminution in the amount of phytate excreted. In other words, the limitation, referred to above, of vitamin D in bringing about Ca absorption is also accompanied by a similar limitation in causing the disappearance of phytate from the intestine.

The second effect of adding phytate to the diet is to increase the Ca excretion in the faeces. Thus an increase in faecal phytate is associated with an increase in faecal Ca (Fig. 58*b*), which means that less Ca is absorbed. In growing animals this may result in the production of rachitic or osteoporotic bones according to the intensity of the effect and the state of the animal at the time. If the vitamin D intake is just sufficient with the basal diet used to prevent rickets, then the addition of phytate, even if it replaces phosphate, will result in frank rickets. With

higher vitamin D supplies, instead of producing rickets, the addition of phytate will tend to produce a more osteoporotic condition of the bone. This action of phytate on bone structure can be seen in Plate XXIV, radiographs of the costochondral junctions. The radiographs show the ribs (*b*) to be thick and osteoporotic while those of (*a*) are thinner and more normal. Both these animals received 20 i.u. vitamin D daily and the osteoporotic appearance of (*b*) is due to the larger calcium-depriving effect of phytate as compared with that of phosphate in the diet of puppy 5S (*a*).

Under some conditions phytate may even be responsible for withdrawal of Ca from the body itself. This was shown experimentally in its grossest form by maintaining fully grown dogs on diets poor in Ca and vitamin D and rich in high phytate containing foods such as oatmeal and maize. (Mellanby, 1937). These animals developed severe osteomalacia and osteoporosis with great bone deformity. Additional vitamin D protected animals on these diets to some extent while vitamin D and a sufficiency of Ca gave full protection. Animals on similar diets in which rice or white flour (poor in phytate) replaced the oatmeal and maize, did not develop these gross deformities although the bones were osteoporotic. Thus it will be seen that dietary phytate has the power not only of immobilising Ca in food but also of indirectly causing its withdrawal from the highly calcified tissues of the body such as bones and promoting its loss through the intestinal tract.

The importance of a high Ca intake with phytate

The practical issue, therefore, is how best to bring about perfect bone formation in growing animals when the diet is relatively rich in phytate, as for instance when it contains abundant cereals such as oatmeal or maize. The objective must be to increase the Ca absorption so that

it can be incorporated to the best advantage in the bone and other tissues. Obviously a sufficiency of vitamin D *is essential, but it is just as important at this stage to* increase the Ca intake. There are two reasons for this. In the first place, under the experimental conditions chosen, calcium is often a limiting factor in the diet. Secondly increasing the Ca makes the vitamin, especially if present in small quantities, much more effective. For instance a puppy which received 5 i.u. of vitamin D_2 and an additional 200 mg. daily of Ca (exp. 20) absorbed and retained a quantity of Ca approximately equal to that retained by another puppy of the same litter getting 100 i.u. of the vitamin without the extra Ca (puppy 81). This *synergistic effect of Ca salts with the anti-rachitic vitamin* has long been known and was described in 1922 when it was found that the calcium retention produced by butter was greatly increased if additional Ca carbonate *or Ca phosphate was present in the food (Mellanby). It* was this fact which formed the basis for emphasising at that time the advantages of giving milk rather than an equivalent amount of butter for the calcification of bones in children.

It might be thought that increasing the calcium would only bring about the formation of more insoluble penta-calcium phytate in the intestine and thereby increase both the phytate and the Ca excretion, with little benefit to the animal. It is, of course, true that if the calcium added is excessive and the P intake low such an effect will be produced, but increasing the Ca intake within physiological limits, although causing a slight increase in phytate excretion, also increases the Ca absorption. The idea that the addition of calcium to the diet causes a great increase in the phytate excretion is probably dependent upon rat experiments, a point which will be referred to in more detail later. So far, however, as the

dog experiments are concerned, it is undoubted that one way, and the most important practical way, of overcoming the Ca immobilising effect of a diet rich in phytate, assuming that there is some vitamin D present in the body or diet, is by increasing the calcium intake. A diet which contains vitamin D and is rich both in calcium and phytate, is compatible with perfect bone formation but this is not the case when the diet is rich in both vitamin D and phytate and relatively (but not absolutely) low in calcium. Increasing the Ca intake when the body and the diet are devoid of vitamin D is of little use since much, or in some cases all, of the extra Ca is excreted together with most of the phytate.

The main fact to be gathered from these experiments is that, once sufficient vitamin D is given, the level of Ca absorption is controlled by the amount of Ca and by the amount and proportion of dietary P given as phytate.

Inconsistency of dog and rat experiments as regards phytate effect

It may be asked why it has taken so many years for the anticalcifying action of cereals to be generally accepted. Much of the criticism which has been directed against the work has depended on the results showing that rickets in rats is controlled by the Ca:P ratio of the diet and the availability of P. Can the present results explain the apparent anomalies between rat and dog investigations? In order to produce rickets in rats with certainty it has been usual to deprive the mother of vitamin D during lactation, give a vitamin D-free diet to the young and use an experimental diet of a high Ca:P ratio. This unnatural dietetic technique and its results have largely dominated the subject of a human disease.

The effect of the complete deprival of vitamin D would certainly tend to increase the significance of the Ca:P ratio

of the diet in the aetiology of rickets for, as was demonstrated in the above dog experiments, phosphate becomes, under such conditions, nearly as anticalcifying, in the sense of preventing Ca absorption, as phytate. The second condition of the rat experiments, namely the high Ca:P ratio (Sherman and Pappenheimer 1921) must also have a special but abnormal significance. Clearly with diets containing four or more parts of Ca to one of P, the factor most powerfully determining the degree of calcification is the availability of the P, for however much Ca is absorbed it cannot be incorporated into growing bone unless there is a sufficiency of P with which to combine. The general effect of adding very large amounts of Ca to the diet is to immobilise by precipitation both the phytate and phosphate P of the diet and prevent its absorption. The significance of P as the limiting factor in experiments on rickets was particularly apparent when Bruce and Callow (1934) showed that with diets of high Ca content phytate in the presence of vitamin D was less effective than phosphate in promoting healing of rickets. The problem of the relative availability of phytate and phosphate in rats was further studied by Krieger and Steenbock (1940) both in the presence and absence of vitamin D with diets the Ca:P ratio of which varied over a wide range. As a measure of availability of the P of phosphate and phytate they estimated the degree of calcification of the bones. They found that, in the absence of vitamin D and with an optimal intake of P and a Ca:P ratio of 1:1 the utilisation of phytate P and phosphate P was not greatly dissimilar. With higher Ca:P ratios of 2:1, 4:1 and 6:1 the availability of the phytate P decreased rapidly, whereas the inorganic phosphate remained available to the optimum extent. When, on the other hand, there was vitamin D in the diet, the effect of altering the Ca:P ratios was not so apparent, for the vitamin improved the utilisation of both phytate

and phosphate P at all Ca:P ratios. Nevertheless even in the presence of vitamin D, phytate P was never so readily available as the inorganic form.

These experiments show that phytate and phosphate have different biological availabilities in rats irrespective of the presence or absence of vitamin D, and that these differences only become evident when the P supply to the growing animals is limited. They do not throw any light, however, on the main point at issue, namely whether phytate in the diet is more anticalcifying than phosphate, i.e. more potent in preventing Ca absorption and utilisation, especially when the Ca:P ratio is less than or approaching 1 and there is at least some reserve of vitamin D available. Green and Mellanby (1928) investigated the effect of such diets on rats and their results indicated that phytate (although not recognised at that time as the offending agent) had a more powerful anticalcifying effect than phosphate. They showed among other things that oatmeal (high phytate) was more rachitic than white flour (low phytate). Unfortunately it has proved impossible to confirm earlier results with any consistency and it must be assumed that some unrecognised condition was present in that work. Many experiments were carried out at that time and the results obtained were so consistent that it is impossible to put them aside as fortuitous. The later discovery of Patwardhan (1937) that the mucous membrane of the intestine of rats is rich in phytase suggests that these animals have a much greater power of hydrolysing phytate to inositol and phosphoric acid than other experimental animals tested and that it is this factor which explains the greater difficulty of depressing bone calcification by phytate in rats. The fact that in rats phytate P only becomes much more difficult to absorb than phosphate *in the presence of a very high Ca intake supports this view.* Possibly in the type of rat used in the work of Green and

Mellanby or under the conditions of their experiments the phytase activity of the gut was abnormally small. However that may be, the evidence indicates that the anti-calcifying effect of phytate is much smaller in the rat than in the dog, and that this difference is probably due to the greater phytase content of the rat intestine.

It is clear, therefore, that if the interpretation of the aetiology of rickets as it occurs in human beings had to depend on past experimental work on rats, it would be a right assumption that the Ca:P ratio and the availability of P of the diet explained the action of cereals in this disease. There would indeed be little or no support for the view that cereals differed in their rickets-producing action and that this was dependent largely on their content of phytate. On the other hand the present experiments have abundantly confirmed those of Harrison and Mellanby and shown that in dogs phytate as compared with phosphate has the specific property of reducing the absorption of Ca, especially in the presence of vitamin D. In the dog experiments the Ca:P ratio has proved to be but a poor indication of the relation of diet to rickets. The vitamin D content of the body is always dominant but the intensity of its action as regards calcification can be modified in one direction by adding Ca and in the other by adding phytate. It has been shown that diets having the same ratios and amounts of Ca and P may have different effects on Ca absorption and bone calcification according to the chemical nature of the P containing substance; again, altering the ratio by increasing the phosphates of the diet often has but little effect on Ca absorption. Indeed, it can be said that amounts of Ca and P in the diet, necessary to produce optimal bone calcification, vary greatly according to the other constituents of the diet and cannot be regarded as having any constant significance. It will be seen that the basis of the ordinary technique used to produce rickets

in rats, namely severe limitation of P and excess of Ca intakes, has only a partial relation to the nutritional condition which produces the human or canine disease where the main determining factor is the absorption of Ca in a sufficient and a correct form and not a deficiency either in the supply or absorption of P. The rat, as an experimental animal in the elucidation of the aetiology of rickets as it occurs in human beings, has in some respect proved a defective guide and has long prevented the acceptance of the fact that some cereals produce defective calcification and that this action is dependent on the phytate content and Ca limitation.

The toxamin or anti-vitamin action of phytate—— phytate and phosphate as chemical analogues

It may be of interest to discuss briefly the anti-calcifying action of phytate in the light of other recent discoveries on the antagonistic action of structural analogues, which have not only greatly illuminated the subject of anti-vitamins in general but have opened up many other biochemical problems, including the important field of chemotherapy. It was stated in the introduction that when the anti-calcifying action of cereals, and especially of oatmeal, was first reported, the hypothetical substance responsible for this action was described as an anti-vitamin but that later the word 'toxamin' was used as being more descriptive of its effects. As the chemical nature of the cereal anti-vitamin was not known at that time, no suggestions as to the basis of the antagonism were possible. The word 'toxamin' was substituted for anti-vitamin in this instance in order to describe a substance present in some cereals which, by interfering with Ca metabolism, had a harmful effect on the body. It was thought at that time that vitamin D could effectively prevent this action. The present work shows that this is not entirely the case but that, although

vitamin D can usually cloak the harmful action of phytate, especially as regards the production of florid rickets, it cannot by itself prevent some anticalcifying effect. Phytate of cereals qualifies for the designation 'toxamin' because it interferes with the action of vitamin D, probably not by direct antagonism to the vitamin, but indirectly by limiting the amount of Ca available upon which the vitamin can work. It is recognised, however, that this explanation may not account for all the actions of phytate in relation to vitamin D.

There are obviously a number of ways in which a vitamin can be prevented from doing its work. It may be destroyed in the intestine, or if present in the food or formed in the gut by microörganisms, it may not be absorbed for some reason, but these instances are not due to the presence of an anti-vitamin. Anti-vitamin action may depend on the presence of a substance in food which is a structural analogue of the vitamin and displaces it from the surface on which it catalyses certain essential chemical reactions. In such an instance, while the anti-vitamin has a structural resemblance to the vitamin, it also has a structural difference in or near an active group of the molecule which prevents it from having the biological action of the natural vitamin. Although this is a widely accepted hypothesis, advanced to explain certain cases of biochemical interference of this type, not only in the case of some vitamins but of other substances having a drug-like action, there are certainly other possible modes of interfering action of structural analogues. For instance, the anti-vitamin may not interfere directly with the vitamin but may act by removing the substrate from the vitamin's sphere of influence. This latter type of action is the more likely to be the main explanation of the present problem of interference of phytate in calcification. For instance, as

regards the main problem of Ca absorption from the gut, the antagonism between phytate and vitamin D is unlikely to be direct. If there were such direct antagonism, a more evident quantitative relationship ought to exist between phytate and vitamin D. As has been shown in experiment 16 above, however, increasing the vitamin D of the diet from 20 i.u. to 1000 i.u. did not further antagonise the power of phytate to reduce Ca absorption from the gut. On the other hand, as also shown above, there was good evidence of a direct antagonism between phytate and phosphate in regard to Ca.

In what form the calcium passes from the gut into the bloodstream is not known but it may be that its absorption involves a reaction of a complex containing Ca and phosphate. In the complete absence of vitamin D from the body, whereas little Ca is absorbed, the absorption of P on the other hand may be relatively large. On the addition of the vitamin to the food the absorption of Ca is immediately resumed and with this the P absorbed is also increased. The assumption therefore is that, under the influence of vitamin D, Ca is absorbed in association with phosphate. The view that the absorption of Ca is the dominant factor in the action of vitamin D on the intestine is in agreement with that of Nicolaysen (1937). He demonstrated that, in rats on a low P diet Ca absorption was greatly reduced in vitamin D deficiency, but that when Ca was low and the P medium, the latter was absorbed to an equal extent both in the presence and absence of the vitamin. He suggested that the reduced absorption of P reported in vitamin D-deficient rats was due to the large amount of unabsorbed Ca in the bowel precipitating the P. It may not be out of place to remark here again that the unphysiological high Ca-low P diets used to produce rickets in rats may be misleading, for in dogs with diets of more

vitamin D can usually cloak the harmful action of phytate, especially as regards the production of florid rickets, it cannot by itself prevent some anticalcifying effect. Phytate of cereals qualifies for the designation 'toxamin' because it interferes with the action of vitamin D, probably not by direct antagonism to the vitamin, but indirectly by limiting the amount of Ca available upon which the vitamin can work. It is recognised, however, that this explanation may not account for all the actions of phytate in relation to vitamin D.

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would have been absorbed if some of the phytate had not been converted to phosphate and there is evidence that this actually happens under these conditions. In experiment 17 it was found that 40 to 60 per cent of the phytate consumed disappeared from the gut. This disappearance of the phytate caused by vitamin D will be described and discussed in the next chapter.

In the complete absence of vitamin D, both from the diet and from the body, very little Ca is absorbed from the alimentary canal. As the vitamin D content of the diet increases, the amount of Ca absorbed increases, but now the antagonism between phytate and phosphate comes into play, the amount of Ca absorbed being lower in animals receiving dietary phytate. Vitamin D, probably by promoting the hydrolysis of phytate to phosphate, reduces this effect, but even large additions of the vitamin do not destroy all the phytate and the Ca absorption remains subnormal. Apart from its action in the gut, phytate appears to have little, if any, effect and vitamin D takes full control, improving the calcification of bones both by depositing a Ca-phosphate complex in osteoid tissue and by reducing the proportion of other organic as compared with inorganic material. Even with large supplies of vitamin D, however, unless additional Ca is given to satisfy the undestroyed phytate in the intestine, the calcification of the bones of animals receiving dietary phytate will remain subnormal as compared with that of animals whose phosphorus is given as phosphate.

There is still one further aspect of phytate and vitamin D relationship which, while obviously a part of the general problem, evades explanation. This is a point which was indicated in experiment 14, namely that dietary phytate seems to impose an increased demand on vitamin D. In that experiment it appeared that phytate hastened the disappear-

natural Ca:P ratios it appears that the reduced P absorption in vitamin D deficiency is due to the fact that when the Ca is not absorbed less P is needed, and not to the precipitation of the P by the large amount of unabsorbed calcium in the gut. Assuming, therefore, that Ca is absorbed in some combination containing phosphate, it follows that phytate by combining with Ca to form the insoluble, quickly precipitated penta-calcium phytate (Hoff-Jørgensen, 1944) effectively reduces the formation of any complex containing the more soluble and more slowly precipitated calcium phosphate and thereby the Ca available for absorption. Here then is an instance of structural analogues, phytate and phosphate, competing for a third substance, Ca, the absorption of which is controlled by a vitamin.

Whereas this part of the antagonism between phytate and phosphate and vitamin D is reasonably clear, it is more difficult to understand how the vitamin prevents the phytate effect becoming complete master of the situation. It does this at least partly by establishing conditions favourable to the breakdown, by hydrolysis, of phytate into phosphate. How powerful this action can be is seen in experiment 17 above, where it was shown that, even when the sole source of calcium in the diet was in the form of Ca phytate, the body absorbed a fair amount of calcium when there was a source of vitamin D in the body or in the food. It is true that the amount absorbed was much less than when the source of dietetic Ca was in the form of Ca phosphate; for instance, in experiment 17 above 218–348 mg. of Ca were absorbed daily in the phosphate animal where practically the only source of Ca was $CaHPO_4$, whereas in the corresponding phytate animal only 85–199 mg. of Ca were absorbed. It is difficult to believe that so much of the Ca of the phytate compound

Chapter XV

THE DISAPPEARANCE OF PHYTATE FROM THE GUT

1. The Increased Disappearance of Phytate on Adding Vitamin D to the Diet

We know from the experiments described above that phytate, unlike inorganic phosphate containing the same amount of P, has a specific effect in reducing calcium retention in the presence of vitamin D and that it does this by reducing calcium absorption. It has also been shown above (experiment 19, page 338) that, as an animal on a vitamin D-free diet loses its reserve of the vitamin in the course of an experiment, there is an increase in the amount of unchanged and unabsorbed phytate which passes along the gut. An experiment directed to the examination of the effect of vitamin D on phytate in the alimentary canal is now described.

EXPERIMENT 21

Object. To determine the effect of two dietary changes on the excretion of phytate P: (1) adding vitamin D_2 to the diet of an animal whose reserves of this substance have been exhausted, and (2) removing vitamin D_2 from the diet of an animal whose body reserves are high.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The food of the young puppies was supplemented by a small quantity of cod-liver oil, so that they would be expected to have moderately good reserves of vitamin D at the beginning of the experiment.

Basal experimental diet:

Oatmeal	45–220 g.
Separated milk powder	25 g.

ance of body reserves of this vitamin. Inorganic phosphate may have a similar action, but, if so, it is of a lower intensity than that of phytate. The explanation of an antagonism of this kind, if confirmed by further work, would require more than the Ca-immobilising effect of phytate, which seems to be the main explanation of the latter's action. The interplay of vitamin D, Ca, inorganic phosphate and phytate is still far from being understood.

cretion of phytate phosphorus throughout the whole experimental period. It is thus clear that the addition of vitamin D_2 to the diet of an animal deficient in this sub-

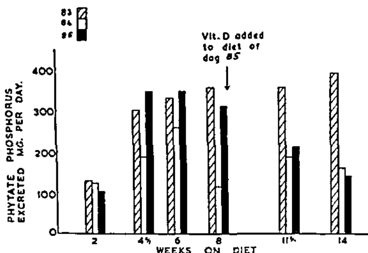


FIG. 67 (Exp 21) Effect on phytate excretion of adding vitamin D_2 to the diet of an animal whose reserves have been depleted

Additions to basal diet

Puppy	Vitamin D_2
83	None
84	500 i.u daily throughout the experiment
85	None until after 8 weeks on diet then given 500 i.u daily by mouth

Note. (1) Puppy 83 (no vitamin D) loses its power to promote the disappearance of phytate.
(2) When vitamin D_2 is added to the diet of 85 the phytate excretion is reduced

stance promptly increases the phytate disappearance so that it approximates to the normal.

In contrast, it is interesting to note how the effect of vitamin D is maintained long after its administration is stopped. Puppy 86 received 17,500 i.u. of vitamin D between the 8th and 13th weeks of life, after which this

Lean meat. .	15 g.
Peanut oil .	10 ml.
NaCl ...	1-2 g.
Baker's yeast .	5% of cereal
Food yeast given after 8½ weeks..	2.5 g.
Ascorbic acid .	5 mg.
$CaCO_3$ . .	0.5 g.

(Ca content: initial 551 mg., final 726 mg.)
(P content: initial 410 mg., final 1190 mg.)

Daily additions to basal diet:

No of puppy	Vitamin D_2
83 (3034)	None.
84 (3038)	500 i u. by mouth throughout the experiment.
85 (3031)	None until after 8 weeks on diet; then given 500 i.u. by mouth.
86 (3036)	500 i.u. by mouth for the first 5 weeks on diet; the supply was then discontinued.

Age at beginning of experiment: 8 weeks.
Duration of experiment: 14½–15 weeks.

Fig. 67 shows that after two weeks on diet, when all the animals would still have pre-experimental reserves of vitamin D, there was but little difference in the amounts of phytate P excreted. After this date all excretions rose, due in part to the increase in the food consumed and therefore to the larger amount of phytate P ingested. But by the fourth week of the experiment there was a definite division, the animal receiving vitamin D (84) excreting less phytate phosphorus (190 mg.) than those receiving no vitamin D (83 and 85) (301 and 352 mg. respectively). This differentiation continued until the eighth week and at that point was large. Then, with the addition of vitamin D_2 to the diet of puppy 85, its phytate excretion fell, and after a further 4 weeks diminished to the level of the puppy having the vitamin throughout (84) and remained low until the experiment was terminated. Puppy 83, which had no additional vitamin D, maintained a consistently higher ex-

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The diet of the litter was supplemented by 2.5 ml. of cod-liver oil per puppy per day until the eighth week of age, i.e. for about 4 weeks.

Basal experimental diet:

Oatmeal..	50–150 g
Separated milk powder	20 g.
Lean meat ..	15 g.
Peanut oil..	10 ml.
NaCl..	1 g.
Baker's yeast	5% of cereal
Ascorbic acid .	5 mg.
Vitamin A acetate.	1000 i.u.

(Ca content: initial 293 mg., final 431 mg.)
(P content: initial 389 mg., final 864 mg.)

Daily additions to basal diet:

No. of puppy	Vitamin D_3
87 (3138)	None.
88 (3139)	200 i.u. per day for 100 days during digestion.
89 (3137)	20,000 i.u. in a single dose given in the food at the beginning of the experiment.

Age at beginning of experiment: 10½ weeks.
Duration of experiment: 14 weeks.

The graph of phytate P excreted at various times during the experiment (Fig. 68a) shows that the effect of the single large dose of vitamin D on phytate disappearance continues even after 13 weeks (compare puppies 87 and 89). Until the 10th week of the experiment puppy 89, which had received the single dose, was able to hydrolyse or absorb as much phytate P as 88, receiving a daily supply of the vitamin, but from this time there was some falling off. It is unlikely that any further supply of the vitamin to 89 would have modified the phytate excretion during the 10 weeks following the single large dose.

Further evidence of the long-continued effect of vita-

substance was omitted from the diet, but, in spite of this, the effect on phytate disappearance was maintained for at least a further 9 weeks, an action reminiscent of prolonged Ca absorption under the same conditions.

The effect of small graded doses of vitamin D_2 on phytate excretion was seen in experiment 20 (Chapter XIV, Fig. 66*a*), where it was shown that 100 i.u. daily (puppy 81) were more effective than 20 i.u. (puppy 79) and that this amount in turn promoted the disappearance of more phytate than 5 i.u. (puppy 77) and much more than when the diet was devoid of the vitamin (puppy 75). Fig. 66*b*, however, showed that, with a higher Ca intake although the addition of vitamin D_2 reduced the excretion of phytate, the amount that disappeared in the puppy receiving 20 i.u. daily was practically the same as that in the puppy receiving 100 i.u. daily. It seems probable that under the conditions of the experiment the 'limiting' amount of vitamin D had been reached, for it was previously shown (experiment 16, Fig. 59*a*) that, above this level, increasing the vitamin D fifty-fold did not significantly alter phytate excretion.

It was next decided to try to obtain information on the storage and economical use of vitamin D, adopting as an indication the amount of dietary phytate that disappeared from the gut; a puppy having a daily dose of the vitamin for 100 days was compared with one receiving the same total amount given in one dose at the beginning of the experiment.

2. The Effect of Vitamin D_2 in a Large Single Dose and in Repeated Small Doses Respectively on the Disappearance of Phytate

EXPERIMENT 22

Object. To compare the effect of vitamin D_2 given respectively in one large dose and many repeated smaller doses on the disappearance of phytate from the gut.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The diet of the litter was supplemented by 2.5 ml. of cod-liver oil per puppy per day until the eighth week of age, i.e. for about 4 weeks.

Basal experimental diet:

Oatmeal	50–150 g.
Separated milk powder	20 g.
Lean meat	15 g.
Peanut oil..	10 ml.
NaCl	1 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i u.

(Ca content: initial 293 mg., final 431 mg)
(P content: initial 389 mg., final 864 mg.)

Daily additions to basal diet:

No. of puppy	Vitamin D_3
87 (3138)	None.
88 (3139)	200 i.u. per day for 100 days during digestion.
89 (3137)	20,000 i.u. in a single dose given in the food at the beginning of the experiment.

Age at beginning of experiment: $10\frac{1}{2}$ weeks.
Duration of experiment: 14 weeks.

The graph of phytate P excreted at various times during the experiment (Fig. 68a) shows that the effect of the single large dose of vitamin D on phytate disappearance continues even after 13 weeks (compare puppies 87 and 89). Until the 10th week of the experiment puppy 89, which had received the single dose, was able to hydrolyse or absorb as much phytate P as 88, receiving a daily supply of the vitamin, but from this time there was some falling off. It is unlikely that any further supply of the vitamin to 89 would have modified the phytate excretion during the 10 weeks following the single large dose.

Further evidence of the long-continued effect of vita-

substance was omitted from the diet, but, in spite of this, the effect on phytate disappearance was maintained for at least a further 9 weeks, an action reminiscent of prolonged Ca absorption under the same conditions.

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It was next decided to try to obtain information on the storage and economical use of vitamin D, adopting as an indication the amount of dietary phytate that disappeared from the gut; a puppy having a daily dose of the vitamin for 100 days was compared with one receiving the same total amount given in one dose at the beginning of the experiment.

2. The Effect of Vitamin D_2 in a Large Single Dose and in Repeated Small Doses Respectively on the Disappearance of Phytate

EXPERIMENT 22

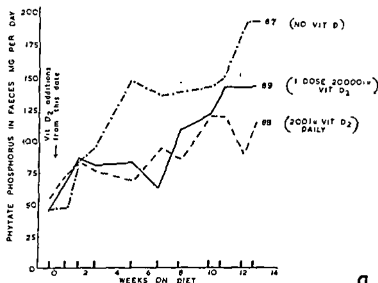
Object. To compare the effect of vitamin D_2 given respectively in one large dose and many repeated smaller doses on the disappearance of phytate from the gut.

min D is seen in Fig. 68b, which shows that puppy 89, receiving the single massive dose, retained approximately the same amount of Ca as puppy 88, receiving the smaller daily dose, just as a large dose of the vitamin is known to allow Ca absorption in children for a long time. (Harnapp 1936, Braulke 1937) The mineral content of the femur shafts of these two animals was approximately equal, both being more than double that of puppy 87 (no vitamin D). Perhaps of greater interest is the fact that the A/R ratios were equal, indicating that during the period of the experiment there was sufficient vitamin D available in puppy 89 (single dose) to maintain bone quality at the same level as in puppy 88 (daily doses). In the case of 87 the absence of dietary vitamin D resulted in a femur shaft of low calcium content and greatly reduced A/R ratio.

No. of puppy	Mineral ash of femur shaft	A/R ratio
87	0.685 g.	0.88
88	1.540 g.	1.28
89	1.794 g.	1.23

In many experiments, especially in those conducted on human subjects, the significance of this slow depletion of vitamin D stores has often been unrecognised. The lack of any obvious effect of adding vitamin D after a short period of a vitamin D-free diet has been interpreted as evidence that vitamin D has no effect on Ca retention. Unless it is known that, in addition to the dietary elimination of vitamin D, the body reserves have been exhausted, the lack of effect means that vitamin D reserves are still exerting a maximum effort both on Ca retention and on phytate disappearance and that further additions of the vitamin may have no effect.

It has long been known that Ca metabolism can be equally well controlled by vitamin D when given parenterally as when given by mouth, and it now appears from



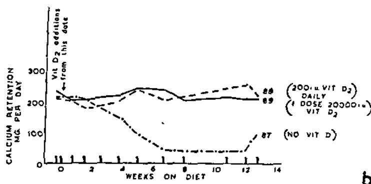


FIG. 68 (Exp. 22). Effect of a single large dose and of repeated small doses of vitamin D_2 on (a) the excretion of phytate phosphorus and (b) the retention of calcium.

Note: In (a), puppy 87 (no vitamin D) soon loses the power to destroy phytate. Puppies 88 and 89 destroy more phytate, and are about equal in this respect for 10 weeks, after which time 89 (large single dose) appears to deal less effectively with phytate than 88 (daily dose). In (b), calcium retention is about equal in puppies 88 (daily dose) and 89 (large single dose) and in both is much higher than in 87 (no vitamin D).

Daily additions to basal diet:

No of puppy	Vitamin D_2
90 (3056)	None.
91 (3057)	20 i.u. vitamin D_2 *by mouth.*
92 (3058)	20 i.u. vitamin D_2 *intravenously.*

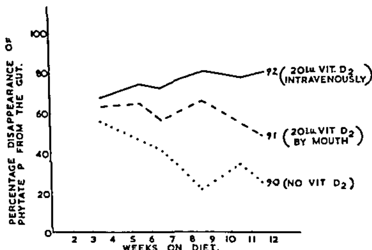


FIG. 69 (Exp. 23). The relative effect of vitamin D_2 given by mouth or intravenously on the percentage disappearance of phytate from the alimentary canal.

Note: 1. That 20 i u vitamin D_2 (91 and 92) has caused an increased disappearance of phytate from the gut, when compared with no vitamin D (90).

2. That intravenous administration of the vitamin has been more effective in promoting this disappearance than has the same quantity by mouth.

Age at beginning of experiment: 7 weeks.

Duration of experiment: 12 weeks.

As the oatmeal of the diet increased in the course of the experiment from 70 to 190 g., the phytate P intake of each puppy increased from 184 to 499 mg.

It is evident from Fig. 69 that after about 4 weeks of the experimental feeding the percentage of phytate dis-

the foregoing experiment that this vitamin also controls phytate changes in a similar way. This action is of interest because, whereas the effect of vitamin D on Ca metabolism affects both Ca absorption and Ca incorporation in bone and one of these may be directly dependent on the other, the changes which phytate undergoes are probably confined to the intestine. The following experiment was therefore made in order to test the relative potency of action of vitamin D when given by mouth and intravenously.

3. Relative Effect of Vitamin D_2 Given by Mouth and Intravenously

EXPERIMENT 23

The object of this experiment was to compare the effects on the disappearance of phytate from the gut of small equal quantities of vitamin D_2 when given by mouth and by intravenous injection respectively.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The food of the young puppies was supplemented by cod-liver oil, so that they would be expected to have some reserves of vitamin D at the beginning of the experiment.

Basal experimental diet:

Oatmeal	70–190 g.
Separated milk powder	30 g.
Lean meat.	20 g.
Peanut oil	10 ml.
NaCl .	1–2 g.
Baker's yeast	5% of cereal
Ascorbic acid .	5 mg.
Vitamin A acetate	1500 i.u.

(Ca content: initial 470 mg., final 548 mg.)
(P content: initial 600 mg., final 1197 mg.)

of phytate from the gut than the same amounts given by mouth.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) Each puppy received 5 ml. daily of cod-liver oil for the 3 weeks prior to the beginning of the experiment.

Basal experimental diet:

Oatmeal	98–200 g.
Separated milk powder	30 g
Lean meat .	15 g
Peanut oil	10 ml
NaCl	1 g
Baker's yeast	5% of cereal
Ascorbic acid	5 mg
Vitamin A acetate	1000 i u

(Ca content: initial 410 mg., final 493 mg)
(P content: initial 582 mg , final 893 mg.)

Daily additions to basal diet:

No of puppy	Vitamin D_2
93 (3247)	20 i.u. vitamin D_2 *by mouth* after food.
94 (3248)	20 i.u. vitamin D_2 *intravenously* after food

Age at beginning of experiment: 8 weeks.
Duration of experiment: 21 weeks.

As can be seen from Fig. 70, there was no difference in the percentage disappearance of phytate phosphorus when vitamin D_2 was given by the two routes. Similar results were obtained from another experiment of the same type. Detailed examination of the methods, times of feeding, etc., appeared to reveal only one difference between experiments 23 and 24. In experiment 23 the vitamin D_2 was given, whether by mouth or intravenously, about 2 hours *before* the daily food, but in the attempt to repeat that experiment it was given about one hour *after* the last of

appearing from the gut was not greatly different in the three animals. The probable reason for this was that in all cases vitamin D reserves were still available (see Chapter XIV), so that the effect of the supplementary vitamin D_2 given to puppies 91 and 92 had not yet become evident. It is probable that, after 6 weeks of the diet, the vitamin D reserves of 90, receiving no dietary D_2, were lost, since the proportion of phytate which disappeared from the gut was greatly reduced at this time. In the case of 91, which received 20 i.u. of the vitamin daily by mouth, the percentage of phytate which disappeared remained fairly high till after the 8th week and then gradually decreased. In 92, the puppy receiving the same amount of the vitamin intravenously, the percentage increased continuously, until during the final 5 weeks of the experiment it remained at about 80 per cent. of the intake.

Two points are worthy of note. The first is that the effect of vitamin D_2, whether given by mouth or intravenously, was definitely to increase the percentage of phytate hydrolysed in or absorbed from the gut, as compared with that lost, when the body stores were exhausted, in the case of the animal not receiving the vitamin. The second point, which was unexpected, is that the vitamin injected into the bloodstream increased the percentage of phytate disappearing from the gut to a greater extent than that given by mouth.

Several attempts were made to confirm this result and one of the experiments carried out for this purpose will now be described.

EXPERIMENT 24

The object here was to repeat experiment 23, which had indicated that vitamin D_2 given in small quantities intravenously was more effective in causing the disappearance

on the phytate in the gut varied according to the time at which it was given in relation to feeding.

Pre-experimental diet. (For maternal diet see Appendix II, p. 432.) The puppies' food was supplemented by cod-liver oil from the age of 3½ weeks until the experiment was begun; hence they had a good body reserve of vitamin D.

Basal experimental diet:

Oatmeal	15–180 g.
Separated milk powder	30 g.
Lean meat	15 g.
Peanut oil	10 ml
NaCl	1–2 g.
Baker's yeast	5% of cereal
Ascorbic acid	5 mg.
Vitamin A acetate	1000 i.u

(Ca content: initial 368 mg., final 450 mg.)
(P content. initial 311 mg, final 880 mg.)

Daily additions to basal diet:

No. of puppy	Vitamin D_2
95 (3278)	None.
96 (3279)	20 i.u. vitamin D_2 by mouth *2 hours before* food.
97 (3280)	20 i.u. vitamin D_2 by mouth *1 hour after* food.
98 (3281)	20 i.u. vitamin D_2 intravenously *2 hours before* food.
99 (3282)	20 i.u vitamin D_2 intravenously *1 hour after* food.

Age at beginning of experiment: 6½ weeks.
Duration of experiment: 17 weeks.

Fig. 71 shows that the apparent discrepancy in the results of the two previous experiments (23 and 24) could be explained by the different conditions under which they were made, for in this combined experiment (25), when the vitamin D_2 was given *before* food (as in experiment 23) that administered by the intravenous route (puppy 98) produced a greater disappearance of phytate than that given by mouth (puppy 96), whereas when the vitamin was given *after* food (as in experiment 24) the two methods

the day's food had been consumed. In both cases the supplement was given in the form of irradiated ergosterol (20 i.u. vitamin D_2) diluted with peanut oil and was either delivered into the mouth from a syringe or injected into a leg vein. It appeared worth while, therefore, to see whether the time of administration of the vitamin in relation to the ingestion of food was the factor which decided whether

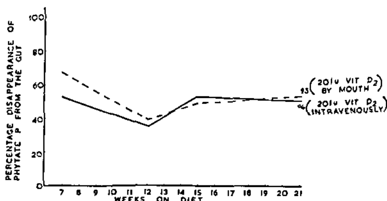


FIG. 70 (Exp. 24). The relative effect of vitamin D_2 given by mouth or intravenously on the percentage disappearance of phytate from the alimentary canal.

Note: That in this experiment when 20 i.u. vitamin D_2 were given after food altering the method of administration (intravenous (94) or by mouth (93)) has had no effect on the disappearance of phytate.

vitamin D_2 would be more or less effective in stimulating the disappearance of phytate from the gut.

In the next experiment the same methods of dilution and administration of the oil to the animals were adopted, but the times of administration in relation to food were varied.

EXPERIMENT 25.

Object. This experiment was made to test whether the effects of giving vitamin D_2 by mouth and intravenously

brings about this curious relationship between vitamin D_2 and phytate disappearance from the gut is that the vitamin, in the quantities used and under these conditions, is less effective in promoting the disappearance of phytate when it is given by mouth 2 hours before food, whereas when given intravenously 2 hours before food or by either method after food, much more phytate disappears. This variation in the effect of vitamin D_2, according *to the time it is given in relation to the feeding of the* animals, has not yet been investigated in detail, but the experiments so far made indicate that with small, possibly sub-optimal doses, the time factor in relation to food is important. This point clearly needs further attention, especially in order to determine how vitamin D in the bloodstream influences the disappearance of phytate from the gut.

Since the ultimate effect of vitamin D on phytate disappearance is probably within the alimentary canal (see p. 380), promoting either the hydrolysis of phytate to phosphate or to some intermediate product or its absorption as phytate, and since the intravenous injection of the vitamin is so effective it seems probable that its action on phytate is indirect. That is to say, the presence of vitamin D in the bloodstream probably causes some reaction within the gut, which in turn promotes the hydrolysis or absorption of phytate (see also p. 390). Possibly vitamin D_2 given by mouth can only be effective after absorption from the alimentary canal into the bloodstream, and it may be that, when it is given before food, some destruction occurs which reduces the amount available for absorption into the bloodstream.

4. Phytate as a Source of Body Phosphorus

The question as to whether the phytate is broken down to phosphate and inositol in the intestine by bacteria or by

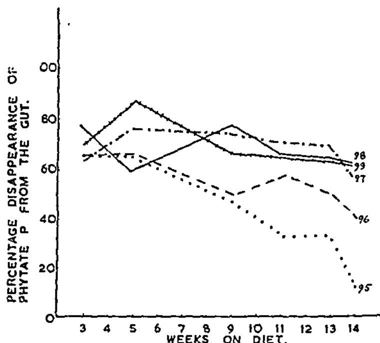


FIG. 71 (Exp. 25) The relative effect of vitamin D_2 given by mouth and intravenously, on the percentage disappearance of phytate from the alimentary canal when the vitamin is given before and after food respectively.

Daily additions to basal diet

Puppy	Vitamin D_2
95	None
96	20 i.u. by mouth before food.
97	20 i u. by mouth after food.
98	20 i.u intravenously before food.
99	20 i.u. intravenously after food.

Note 1. 20 i.u. vitamin D_2 given intravenously *before* food (98) caused a greater disappearance of phytate than when given by mouth (96) (cf Fig. 69).

2. When the vitamin was given *after* food the two methods of administration (97 by mouth, 99 intravenously) produced similar results (cf. Fig. 70).

did not produce significantly different effects (puppies 97 and 99). It will also be seen that the outstanding fact which

the gut of this dog could have come from the non-phytate phosphorus compounds in the food. There is, therefore, no clear evidence that phytate was available to this animal.

In the case of the puppy receiving vitamin D (91), however, neither of the above conditions holds. In the first instance, 783 mg. P (total) were absorbed from the gut, and since only 622 mg. P were given in the food as non-phytate phosphorus compounds, 161 mg. (i.e. 783 − 622) of phosphorus given as phytate must have been absorbed from the alimentary canal. Again, 308 mg. of phytate phosphorus disappeared from the gut and yet only 147 mg. of phosphate were found in the faeces. Therefore, 161 mg. of phytate phosphorus must have been absorbed in some form and become available to the animal. In the presence of vitamin D, therefore, there is evidence that the phosphorus of phytate is available to the dog. This may also be the case in the puppy on the D-deficient diet (90) to a much less extent, although experiment 23 provides no conclusive evidence on this point. Even in the case of the animal receiving vitamin D, there is no evidence as to how the phytate phosphorus becomes available, i.e. whether as phytate itself or only after conversion to phosphoric acid or intermediate products. This question of the availability of phytate phosphorus has been demonstrated in rats by Krieger, Bunkfeldt and Steenbock, (1940 (a)). They showed that adding P as phytic acid to a basal diet rich in Ca but low in P improved calcification of bone. When in their experiments dietary phosphorus was raised to 0.297%, most of which was present as phytic acid, they found that calcification approached that of the control animals receiving the same amount of P in the inorganic form. It thus seems clear that P fed as phytic acid can be utilized by the body both in dogs and rats.

Let us now examine the evidence bearing on the question of the breakdown of phytate to phosphate and inositol.

a non-bacterial enzyme will be discussed later. For the present, evidence bearing on the availability of the phytate to the body and the form in which it is absorbed will be considered.

(a) *Availability*

That phytate phosphorus, either as such or as the inorganic product of its hydrolysis, can be absorbed and made available to the dog is seen in Experiment 23. The figures

TABLE 33 (See Experiment 23 for details)

Availability of phytate phosphorus

No. of puppy	Vitamin D_2	Mg. phosphorus per day								
		In food			In faeces			Disappeared from gut		
		Total	Phytate	Phosphate*	Total	Phytate	Phosphate*	Total	Phytate	Phosphate*
90	None	1082	460	622	570	360	210	512	100	412
91	20 i.u. by mouth	1082	460	622	299	152	147	783	308	475

* Includes any organic phosphorus which does not react as phytic acid phosphorus.

shown in Table 33 give the phosphorus balances in two of the puppies, 90 (D-deficient diet) and 91 (20 i.u. vitamin D_2), after $8\frac{1}{2}$ weeks on diet.

In the puppy on the D-deficient diet (90) all the 100 mg. of phytate phosphorus which disappeared from the gut could be accounted for on the assumption that it had been hydrolysed by bacteria in the large intestine and excreted, since the 210 mg. of phosphate in the faeces might contain the product of this hydrolysis. Likewise all the 512 mg. of phosphorus-containing substances absorbed from

absorbed 283 mg. of phosphorus as phosphate and 216 mg. as phytate. Since no phytate phosphorus was excreted via the urine, and yet the total phosphorus of the urine was greater by 225 mg. than the phosphate fed, it follows that this phosphorus at least must represent hydrolysed phytate. Even then, the 91 mg. of phosphorus retained by this puppy would still have to be accounted for by the phytate P fed. That is to say, that 216 mg. of P fed as phytate have been utilized by the animal, being either hydrolysed and excreted, or retained, possibly in the form of phytate, but more likely as the inorganic phosphate resulting from hydrolysis. A similar argument holds good also for puppy 61.

No stores of phytate phosphorus have been observed in dogs. Rapoport (1940) found phytic acid in the red cells of certain avian blood, but the amounts were small. Blood of the dog and of some other animals as well as birds has been tested in this laboratory by the Leva and Rapoport (1941) technique and, although it has been confirmed that phytate is present in bird's blood, none has been found in the mammalian bloods tested. Thus it seems unlikely that phosphorus can be absorbed and stored in the body as phytate and it is probable that it is hydrolysed either in the intestine before absorption or in the body immediately after absorption. The lack of evidence of the presence of phytate in the animal's body suggests that the hydrolysis takes place in the alimentary canal, but the present figures do not prove this and it is possible that the phytate was first absorbed and then hydrolysed. It can be stated, however, on the basis of the balance figures of these experiments on dogs, that the change of phytate to phosphate is physiological and takes place normally.

It has now been shown (1) that the phosphorus of phytate is available to the animal receiving vitamin D_2, i.e

(b) *The breakdown of phytate to phosphate*

Here the evidence bearing on the possible conversion of phytate to phosphate will be considered, for, although many workers assume that this hydrolytic change takes place, there is little or no evidence, except in the rat, that it does actually occur inside the gut.

TABLE 34 (See Experiment 16, p. 320 for details)

No. of puppy	Vitamin D_2 in food (i u.)	Mg phosphorus per day										
		In food			In faeces			Dis-appeared from gut		In urine		Re-tained
		Total	Phytate	Phosphate	Total	Phytate	Phosphate	Total	Phytate	Total	Phosphate	Total
63	1000	837	554	283	338	177	161	499	377	408	408	91
61	100	837	554	283	309	135	174	528	419	436	436	92

The figures given in Table 34 show the phosphorus present in the food, faeces and urine of puppies 63 and 61 (experiment 16) after 14 weeks on the experimental diet.

Considering in the first place puppy 63, as there were only 161 mg. of phosphate P in the faeces, the 377 mg. of phytate phosphorus which have disappeared from the gut cannot all have been hydrolysed to phosphate in the large intestine and excreted in the faeces. Some or all of it must have been absorbed. Table 34 also indicates that 499 mg. (837–338) of total phosphorus have been absorbed. Since only 283 mg. of this 499 mg. could have come from phosphate in the food, 216 mg. of the absorbed phosphorus must have come from phytate.

Even assuming, therefore, that the phosphate found in the faeces has been converted from phytate within the large gut, where it would not be absorbed, puppy 63 has

TABLE 35 (See Experiment 20 for details)

The relative amounts of phytate phosphorus and total phosphorus absorbed under the influence of vitamin D

Note: These figures were obtained after approximately 10 weeks of the experiment when the vitamin D reserves of puppies 75 and 76, which received no dietary supplement of the vitamin, would be almost, if not entirely, exhausted. Puppies 77 and 78 received 5 i.u. vitamin D daily; 79 and 80 received 20 i.u. vitamin D daily; 81 and 82 received 100 i.u. vitamin D daily. Puppies 76, 78, 80 and 82 received 200 mg of additional Ca as $CaCO_3$.

No. of puppy	Vitamin D added to basal diet, i.u.	Total phosphorus absorbed, mg	Phytate phosphorus disappeared from the gut, mg
77	5	397	105
75	0	337	48
Difference	5	60	57
79	20	427	129
75	0	337	48
Difference	20	90	81
81	100	550	162
75	0	337	48
Difference	100	213	114
78	5	394	81
76	0	370	24
Difference	5	24	57
80	20	452	146
76	0	370	24
Difference	20	82	122
82	100	427	110
76	0	370	24
Difference	100	57	86

that it can be absorbed from the alimentary canal in some form, even when given in the food as calcium phytate (experiment 17), and (2) that the change from phytate to phosphate is a physiological one in the sense that phosphorus given in the food as phytate can appear as phosphate in the urine, but where the hydrolytic change takes place has not yet been demonstrated.

5. Is Phytate Hydrolysed in the Gut and Absorbed?

The question of the possible hydrolysis of phytate to phosphate in the gut will now be examined. Evidence on this question can be considered from several angles: (a) direct or indirect evidence from metabolism and other biochemical observations on phytate behaviour, (b) the transformation of phytate to phosphate in vitro by enzymic preparations made from the mucous membrane of the alimentary canal or its contents, or by bacteria either in the small or large intestine.

(a) *Evidence from metabolic observations*

It was stated above that no stores of phytate have been reported in the dog's body. This fact, coupled with the apparent absence of the compound from its blood, suggests that the hydrolysis of phytate does not take place after absorption from the alimentary canal. If phytate is hydrolysed to phosphate before absorption, and there is a certain amount of evidence supporting this view, it would be expected that the phosphate produced from phytate would be as available for absorption as the other non-phytate phosphorus compounds and that factors which stimulate the disappearance of phytate, such as vitamin D (see pages 363), would also increase the absorption of non-phytate phosphorus. This is roughly true, as will be seen in Table 35 taken from Experiment 20.

TABLE 36 (Experiment 25)

Variations in the distribution of faecal phosphorus when vitamin D_2 is given by mouth or intravenously

No. of puppy	Vitamin D_2, i.u.	Mg. phosphorus per day							
		In food			In faeces			Absorbed	
		Total	Phytate	Phosphate*	Total	Phytate	Phosphate*	Calcium	Phosphorus
		After 9 weeks							
95	None	793	333	460	303	174	129	49	490
96	20 by mouth before food	793	333	460	294	163	131	291	499
98	20 intravenously before food	793	333	460	260	71	189	325	533
97	20 by mouth after food	793	333	460	270	83	187	317	523
99	20 intravenously after food	793	333	460	324	110	214	316	469
		After 13 weeks							
95	None	880	400	480	365	264	101	−32	515
96	20 by mouth before food	880	400	480	328	196	132	292	552
98	20 intravenously before food	880	400	480	341	141	200	332	539
97	20 by mouth after food	880	400	480	245	122	123	335	635
99	20 intravenously after food	880	400	480	281	143	138	343	599
		After 14 weeks							
95	None	880	400	480	434	348	86	−80	446
96	20 by mouth before food	880	400	480	390	234	156	256	490
98	20 intravenously before food	880	400	480	435	147	288	283	445
97	20 by mouth after food	880	400	480	386	169	217	294	494
99	20 intravenously after food	880	400	480	230	152	78	311	650

* Includes any organic phosphorus which does not react as phytate phosphorus.

From these figures it will be seen that, as the absorption of phosphorus compounds from the alimentary canal is quantitatively influenced by the vitamin D content of the diet, so also is the disappearance of phytate similarly affected. It is difficult to avoid the conclusion that the additional P absorbed under the influence of vitamin D has been provided, in part at least, by phytate. Since a large part of this total phosphorus absorbed was certainly inorganic phosphate, it might be considered likely that phosphate was also the breakdown product of phytate offered for absorption. While this evidence is suggestive, it is not a proof of such a change.

Is there ever an occasion when phytate disappearance from the gut is accompanied by an increase in phosphate in the faeces, whilst at the same time there is abundant absorption of phosphorus from the intestine? If there were not a large absorption of phosphorus under such circumstances, it might simply indicate either a back excretion of phosphate through the large intestine, as seems to happen under some conditions, or a breakdown of phytate to phosphate in this region by bacteria. An instance will now be given where there was a large breakdown of phytate occurring simultaneously with a large absorption of phosphorus from the gut. When, in experiments 23 and 25 (see pages 368, 372), small quantities (20 i.u.) of vitamin D_2 were injected intravenously before food, it will be seen in Tables 36 and 37 that increased disappearance of phytate phosphorus from the gut was accompanied by *an increase* in the phosphate of the faeces. Since the amount of total phosphorus absorbed and retained by the animal was roughly the same as when vitamin D_2 was given by mouth, it can only be inferred that vitamin D_2 given in the blood-stream in this way caused a greater breakdown of phytate to phosphate in the gut, and that, since the body could only absorb and utilize a certain amount, the excess was passed along the intestine and excreted.

TABLE 36 (Experiment 25)

Variations in the distribution of faecal phosphorus when vitamin D_2 is given by mouth or intravenously

No. of puppy	Vitamin D_2, i.u.	Mg. phosphorus per day							
		In food			In faeces			Absorbed	
		Total	Phytate	Phosphate*	Total	Phytate	Phosphate*	Calcium	Phosphorus
	After 9 weeks								
95	None	793	333	460	303	174	129	49	490
96	20 by mouth before food	793	333	460	294	163	131	291	499
98	20 intravenously before food	793	333	460	260	71	189	325	533
97	20 by mouth after food	793	333	460	270	83	187	317	523
99	20 intravenously after food	793	333	460	324	110	214	316	469
	After 13 weeks								
95	None	880	400	480	365	264	101	−32	515
96	20 by mouth before food	880	400	480	328	196	132	292	552
98	20 intravenously before food	880	400	480	341	141	200	332	539
97	20 by mouth after food	880	400	480	245	122	123	335	635
99	20 intravenously after food	880	400	480	281	143	138	343	599
	After 14 weeks								
95	None	880	400	480	434	348	86	−80	446
96	20 by mouth before food	880	400	480	390	234	156	256	490
98	20 intravenously before food	880	400	480	435	147	288	283	445
97	20 by mouth after food	880	400	480	386	169	217	294	494
99	20 intravenously after food	880	400	480	230	152	78	311	650

* Includes any organic phosphorus which does not react as phytate phosphorus.

It will be seen from Table 36 that, although in each test vitamin D_2, whether given by mouth or intravenously, resulted in *absorption of approximately the* same amount of calcium and phosphorus, the amount of phytate phosphorus in the faeces varied. These amounts were approximately equal in the puppies receiving their supplement after food, i.e. 97 and 99, but when the vitamin was given before food, as in the case of 96 and 98, that given by the intravenous route (98) was more effective, not only in promoting the disappearance of phytate from the gut, but also in increasing the amount of inorganic phosphate in *the faeces. If the action of vitamin* D was *merely to stimulate* the destruction of phytate by bacterial decomposition, it would be reasonable to expect a greater effect when the vitamin was given via the alimentary canal, but this does not appear to be the case and more phytate disappeared after the intravenous injection.

Another example of the more effective hydrolysis of phytate in the gut when vitamin D is given by intravenous injection than when given in the same amount by mouth can be seen in Experiment 23.

Although there may be another explanation of these results, it is difficult to avoid the conclusion that, when administered before food, the intravenous injection of vitamin D into puppy 98 (Experiment 25) and puppy 92 (Experiment 23) has in some way increased the breakdown of phytate *to phosphate as* compared with that *produced* by vitamin D given by mouth, and that only a portion of the extra phosphate could be absorbed, the rest appearing in the faeces. It might be suggested that the effect of the intravenous vitamin D has been to increase the bacterial breakdown of phytate to phosphate in the large gut where it could not be absorbed, but this explanation does not seem likely.

It is probable that the general effect of vitamin D_2 on

phytate metabolism is the same whether the vitamin acts via the bloodstream or via the food and it may be that in animals whose body reserves of vitamin D are small or

TABLE 37 (Experiment 23)

Variations in the distribution of faecal phosphorus when vitamin D_2 is given by mouth or intravenously

No. of puppy	Vitamin D_2, i.u.	Mg phosphorus per day							
		In food			In faeces			Absorbed	
		Total	Phytate	Phosphate*	Total	Phytate	Phosphate*	Calcium	Phosphorus
		After 6½ weeks							
90	None	1012	420	592	438	244	194	72	574
91	20 by mouth	1012	420	592	313	185	128	316	699
92	20 intravenously	1012	420	592	304	104	200	322	708
		After 8½ weeks							
90	None	1082	460	622	570	360	210	−47	512
91†	20 by mouth	1082	460	622	299	152	147	311	783
92	20 intravenously	1082	460	622	398	82	316	319	684
		After 10½ weeks							
90	None	1152	499	653	510	320	190	75	642
91†	20 by mouth	1152	499	653	515	233	282	206	637
92	20 intravenously	1152	499	653	641	100	541	233	511

* Includes any organic phosphate which does not react as phytate phosphorus.

† The P excreted via the urine was high in these samples so that the P retained was approximately equal to that retained by 92.

absent, vitamin D_2 in the food may have to be first absorbed into the bloodstream to be effective. A possible mode of action of this type would be analogous to the effect of secretin on digestive processes, and it may be

that, just as secretin injected intravenously causes a large flow of pancreatic juice into the intestine, so also the intravenous injection of vitamin D causes an increased secretion of phytase somewhere into the intestine and that this acts on the phytate in the food. But, whatever the position, here is evidence that phytate is broken down to phosphate in the gut. It has only been possible to get such evidence when the breakdown was excessive and produced more phosphate than could be absorbed. But evidence from another direction supports the idea that phytate which 'disappears' from the gut is mostly broken down to phosphate and that some of it is then absorbed.

(*b*) *Bacterial or Naturally Secreted Phytase*

The evidence in this section is of a more direct kind and shows that a breakdown of phytate to phosphate can be produced *in vitro* by the gut contents and that the process is probably not bacterial or due to an enzyme ingested with the food as suggested by some workers. (Plimmer 1913, Starkenstein 1914, Lowe and Steenbock 1936b, Møllgaard et al. 1946).

Hydrolysis of sodium phytate by intestinal contents. Fresh dog faeces were mixed with ten times their weight of distilled water, allowed to stand for 1 hour, centrifuged and the centrifugate Seitz-filtered. The filtrate was mixed with Na phytate and buffer solutions and incubated for 24 hours. A definite destruction of phytate occurred over a wide range, pH 5 to 6.5, showing that the optimum pH is considerably lower than for the rat (pH 7.6; see p. 270).

It has been suggested by Møllgaard et al. (1946) that the phytase activity of the intestinal contents might be due to the presence of this enzyme in the food eaten. This is certainly probable when uncooked cereals are eaten, but in the experiments described above all the food was cooked and the phytase destroyed. The cereal, separated milk

powder and meat were cooked for 1½ hours at ½ lb. steam pressure, the yeast was boiled for 5 minutes, and the peanut oil was heated, in some cases to 200°C. for 30 minutes. The only constituents of the diet which were not heat-treated were the ascorbic acid, vitamin A acetate and vitamin D_2, and it is unlikely that any of these could directly exert any phytase action. Tests were made to see whether vitamin D_2 had any such action or whether *in vitro* it influenced phytase activity when added to intestinal contents, but the results were negative. It is therefore probable that in the present experiments the phytase in the intestinal contents was a normal part of the animals' digestive processes and did not depend on the phytase activity of the food itself.

The question now arose as to where phytase acted in the intestine of these puppies. It has been mentioned above (Chapter XIII, p. 271) that it had not been possible to demonstrate the presence of phytase in the mucous membrane of the alimentary canal of the dog, as had been possible in the case of the rat. Again, the phytase above discussed was actually found in the faeces and, if this were the only place where this enzyme were found in the dog, it would indicate that phytase activity could only take place in the large intestine, possibly as the result of bacterial action, but if so, it was unlikely that the products of hydrolysis, namely inorganic phosphate and inositol, would then be absorbed, as had been shown to happen to the former of these substances by balance experiments.

Obviously it was desirable, not only to test for the presence of phytase in all parts of the alimentary canal, but also to estimate the relative potencies of its action, but these could not be determined for certain technical reasons. It has, however, been found that there is a phytase in the contents of the alimentary canal of the dog from the duodenum to the rectum, and the evidence suggests that

the greatest concentrations are present in the duodenal and rectal contents.

It was decided next to see whether the phytase effect of intestinal contents could be ascribed to bacteria, although the fact that the phytase activity was potent in the duodenum suggested that the hydrolytic change was not primarily bacterial in origin. A puppy, the faeces of which were known to have a fairly high phytase activity, was killed and cultures were made from the duodenum. This site was chosen for the test as it was considered that if the phytase found to be present was bacterial in origin, then cultures from this region would contain fewer types of micro-organism than those from the rectum, and that it would therefore be easier to isolate those micro-organisms responsible for the phytase activity of the intestinal contents. All types of bacteria present were not cultured, but several were grown both in ordinary media and in those to which sodium phytate had been added. Good growth was obtained but no reduction in the phytate P content of the media occurred, nor were the bacteria which were first grown in normal media and then tested in media containing phytate found capable of hydrolysing this substance. The bacteria isolated from intestinal contents were also added singly and together to boiled duodenal contents and here again no hydrolysis of a sodium phytate substrate occurred. All direct attempts to trace the phytase activity to bacteria failed and it was therefore decided to extend the enquiry to see whether the intestinal contents contained a phytase independent of all micro-organisms.

Demonstration of phytase activity of a sterile extract of dog faeces. It will now be shown that intestinal contents (in this case faeces) when freed from all micro-organisms (so far as is known) still retain good phytase activity. 33 g. of fresh faeces were thoroughly mixed and evenly suspended in 350 ml. H_2O, and 60 ml. were set aside as solution A.

The remainder was centrifuged at 4000 r.p.m. for 10 minutes, and a sample set aside as solution B: the rest was filtered through a Seitz filter; part of the filtrate was called solution C and the rest passed through a Berkefeld filter and called solution D. These solutions were tested for bac-

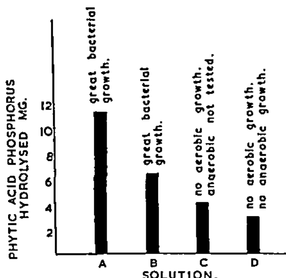


FIG. 72. Relative amounts of phytate hydrolysed by extracts of faeces before and after removal of bacteria.

Note: Although removing bacteria has resulted in a loss of phytase activity, the sterile solution is still active, indicating the probability that the phytase was naturally present in the gut and that the bacteria are not essential to the hydrolysis.

terial contents. Solution D gave no evidence of growth, either under aerobic or anaerobic conditions.

The effect of incubating 25 ml. each of solutions A, B, C and D with 25 ml. of a veronal-Na phytate mixture for 24 hours is shown in Fig. 72. It will be seen that, although solutions A and B, which gave the greatest growth, also had the highest phytase activity, yet C and D, which gave no evidence of bacteria, were fairly rich in this en-

zyme and the reduction in their enzyme activity migh well have been due to absorption on the Seitz or Berkefelc filter.

Thus it is clear that, although treatment by filtration to sterilise the faeces suspension causes a reduction in the phytase activity, it is possible to obtain sterile solutions which are capable of hydrolysing phytate. This evidence is not conclusive, in that the enzyme might be produced by bacteria within the alimentary canal, but this seems unlikely in view of the other experiments described, especially those showing the presence of phytase activity in the duodenal contents.

Having obtained good but possibly not conclusive evidence that the phytase activity of intestinal contents was independent of bacteria, it was clear that a new field of enzyme physiology presented itself for study. It seemed possible that phytase was produced in the intestine by some alimentary secretion and a few attempts were made to find this source, but these were unsuccessful. It also seemed possible from the foregoing results that vitamin D was associated with either the degree of activity or the method of secretion, or both, of the enzyme. It was hoped to be able to offer *in vitro* evidence that the presence of vitamin D in an animal influenced the amount of intestinal phytase secreted, but this has not yet been possible as the problem has proved more difficult than was expected owing to complicated variations in the calcium and in the inorganic phosphate and phytate contents of the intestine produced by the presence of the vitamin which greatly modify the activity of phytase. All that can be claimed is that vitamin D greatly influences the hydrolysis of phytate in the gut, but whether it does this by increasing the amount present or by modifying the intestinal conditions so as to allow increased activity of the phytase or by both methods is not established.

Discussion

This chapter deals with the nature and significance of the changes which dietary phytate undergoes in the alimentary canal. In the previous chapter it was shown that phytate which is excreted unchanged in the faeces bears a quantitative relation to Ca similarly excreted and that it is highly probable that Ca and phytate are usually combined on such occasions to form an insoluble salt. Now, however, it is necessary to consider the fate of that part of the ingested phytate which disappears. One factor seen to be of prime significance in this change is the presence of vitamin D_2 in the body. Since the main property of vitamin D_2 is to promote absorption of Ca and since, as shown above, there are many factors in common between Ca absorption and phytate disappearance, it is probable that vitamin D activity, Ca absorption and phytate disappearance are associated in one common action.

It is unlikely that phytate which disappears is absorbed as such from the gut, although it is true that the P of phytate is absorbed, and also that more is absorbed when Ca absorption is higher. It is also shown in chapter XIV that, even when most of the Ca in the diet is in the form of Ca phytate, a good deal of Ca and about half of the phytate P is absorbed when vitamin D is present. In spite of this fact it is difficult to believe that phytate itself passes into the bloodstream. None has ever been found in the urine in the course of this work, even when the diet is rich in the substance, and similar negative results have been obtained as regards the blood and other mammalian tissues. It is true that certain avian red blood corpuscles (Rapoport) contain phytate, but examination of dogs' red cells have failed to reveal its presence there. It is also unlikely that phytate obtains access to the bloodstream because in solution its power to precipitate Ca is so great that it is a very toxic substance. Indeed,

the only safe form in which it could be absorbed would be as a Ca salt and this is so insoluble at the body pH that it would probably be unavailable, even if harmless in this form. Its absence from mammalian fluids and tissues stands out in contrast to the large amount of phytate in the form of Ca and magnesium salts which are found in vegetable life.

Although there is no direct evidence of phytate itself being absorbed into the bloodstream there is some evidence, as shown above, that it is hydrolysed in the alimentary canal of puppies, to inorganic phosphate. It seems most probable therefore that dietary phytate which disappears from the gut is first hydrolysed to phosphate and that some is absorbed in this form and some is excreted as phosphate in the faeces.

Attention has been given above to the question as to how phytate is hydrolysed and the view has been adopted that this is due to a phytase normally secreted into the alimentary canal. The old idea that this is brought about by bacterial action is probably untrue. No pure culture or mixture of microörganisms has been isolated from the alimentary contents capable of hydrolysing phytate. On the other hand, watery suspensions of faecal contents have been freed from detectable bacteria by Seitz filtration and found to hydrolyse this substance. Watery suspensions have also been made of duodenal contents where microorganisms are not so numerous and of other parts of the alimentary canal and yet found to have good phytase activity. Thus there is definite support for the view that the enzyme phytase is a normal constituent of the contents of the gut from the duodenum downwards.

Although it is probable that the enzyme phytase is present in the gut contents as the result of a natural secretion, attempts to establish its origin have not yet been successful. It is not present in the mucous membrane

of the alimentary canal in dogs, as it certainly is in that of rats, as was first shown by Patwardhan (1937), nor has any success been obtained in tracing its presence to the gastric juice, to the pancreatic juice or to the bile. Other experiments were made in which extracts of the mucous membrane of the alimentary canal of dogs were added to the gastric juice, bile and pancreatic juice respectively but no evidence of phytase activity was obtained. There remains the possibility that phytase is excreted in the succus entericus but this point has not yet been investigated. All that is certain is that phytase is present in the gut contents of dogs from the duodenum downwards and that it is not present under the conditions tested in the mucous membrane of the alimentary canal (p.271). It is also *probable that this enzyme is not bacterial in origin* and that in the cases investigated above it does not come from the food.

The balance of evidence is that a variable part of ingested phytate, the amount being dependent on the composition of the diet, is hydrolysed to phosphate in the alimentary canal by a phytase which is a physiological component of the intestinal juice of the dog, also that the inositol and the phosphate which are the products of the hydrolysis can be absorbed into the bloodstream if circumstances are favourable, but some of the phosphate may be unabsorbed and excreted. That part of the phytate not absorbed is excreted, probably in conjunction with Ca as an insoluble salt, thereby reducing the amount of this element available to the animal.

How the presence of vitamin D and a reduction of dietary Ca respectively increase the disappearance of phytate from the gut is not known. Each of these may increase the phytase secreted into the alimentary canal, but it is just as likely that there is no increase in amount of phytase and that it is the changed chemical conditions

which allow greater activity of the enzyme present. Thus, the absence of the vitamin raises the Ca of the intestinal contents by suppressing its absorption and it may well be that phytase activity is directly related to the Ca content of the fluid in which it acts and is lowered by a higher concentration of Ca. An explanation of this kind would both unify and simplify this particular part of the problem of phytate disappearance.

Chapter XVI

PHYTATE AND CA ABSORPTION IN MAN

Much of the foregoing account of studies made on animals, and especially dogs, to demonstrate and determine the degree of anti-calcifying or rachitogenic action of different cereals and cereal products must have seemed academic and removed from practical experience. In the present chapter a description will be given of experimental work showing that cereals, and in particular their constituent phytate, also depress calcium absorption and metabolism in human beings. Most of the work, which is of good quality, considering the great difficulties in planning and carrying out on man investigations of this kind, has been done by McCance and Widdowson (1942 a and b) at Cambridge University, and the results must disperse any doubts there may be as to the practical significance of the problem. Any class of foodstuff, which in communities such as those of the U. S. A. and of Britain provides about 40 per cent. of the energy of the diet and in some tropical countries a much higher proportion, must be regarded as important. When, as is evident from the foregoing account, these substances have a powerful but varied effect in interfering with bone and tooth calcification, and indeed with calcium metabolism in general and therefore with normal growth and health, it can be realised how important the study of this action is and how especially necessary it is to determine those conditions which prevent or mitigate this harmful effect of cereals on the animal and human body.

The human investigations to be described consisted mainly of balance experiments on adults, and the results are clear cut. It is necessary, however, to remember that

results obtained on adults do not bring home the even more significant effect of cereals on younger people. The anticalcifying or rachitogenic action of cereals is of greatest importance in infancy, childhood and adolescence, since it is at these times, when the bones, teeth and other organs are growing rapidly and thereby require a large calcium retention for incorporation into their structure, that the depressant effect of cereals may be most harmful. Tests made by Hoff-Jørgensen and his colleagues, described later, show that phytate also acts as as anticalcifying agent in infants and children. Thus it will be seen that the results of the work on men, women and children are in close accord with the findings in rickets and balance experiments on growing dogs described in Chapter XIV. It seems, therefore, that the more detailed data obtained on puppies are also a good and probably a true representation of the effect of high cereal diets on man.

Following the demonstration by Harrison and Mellanby in 1939 that phytate is the factor responsible wholly or mainly for the rachitogenic action of cereals, and especially oatmeal, in young dogs, McCance and Widdowson (1942 a) carried out on human subjects a feeding test, one object of which was to see the effect of phytate, either in flour or as a pure substance added to the diet, on calcium and other mineral metabolism. Balance experiments were made over a period of 9 months on 5 healthy men and 5 healthy women living on diets, 40–50 per cent. of the calories of which were provided by wheat flours of the following types: 69% extraction, 92% extraction, 69% extraction fortified with calcium carbonate or mono-hydrogen phosphate, 92% extraction fortified with the same salts, 69% extraction with the addition of sodium phytate, 92% extraction with a supplement of 2000 i.u. calciferol (vitamin D_2) per day.

It is of interest to note that in designing the balance tests, McCance and Widdowson aimed at solving the following problems:

"1. Do men and women absorb calcium less freely from brown than from white flour dietaries?

2. If this should prove to be the case,
   (a) Is phytic acid the noxious agent, and, if so, has it any effect on other metals?
   (b) Will vitamin D restore the Ca absorption and balances to their white bread levels?
   (c) Will the fortification of wheatmeal flour with calcium salts overcome its bad effect on the absorption of this element and if so, (α) what is the best salt to use? (β) How much should be added?"

Before considering the answers given by McCance and Widdowson, it may be worth while discussing briefly those that would be expected from the results of the animal experiments described in Chapters XIV and XV.

On the basis of the relative rachitogenic actions of white and brown bread on growing dogs (Mellanby, 1925), it would be expected that the calcium of brown bread would be less freely absorbed that that of white bread and, from the early work described in Chapter XIV, that phytate would be an effective agent in depressing the absorption of calcium, even in the presence of vitamin D. It would, however, have been difficult to foresee the effect in man of other metals, except possibly of magnesium, and even this guess would have been based on the fact that magnesium as well as calcium is associated in nature with phytic acid in the form of insoluble phytin. It would have been foretold with certainty that the fortification of wheatmeal flour with calcium salts would antagonise its adverse effects on the absorption of this element. This action of added calcium salts has often been referred to

in earlier chapters, and indeed in the earliest papers on the anticalcifying effect of cereals (Mellanby, 1922, 1925), and has been related to the phytate content of cereals (Harrison and Mellanby, 1939). However, it must also be emphasised that this antagonistic effect of calcium salts to phytate depends largely on there being some vitamin D either in the food or in the body. This need of a reserve of vitamin D in the body to make calcium effective is *demonstrated clearly in Experiment 18, Chapter XIV.* The further deduction can be made that, if there is no body store of vitamin D, additional calcium is useless, since in dogs most is excreted in the faeces and lost, even when substantial quantities are given. Thus the answer to question 2 (c) of McCance and Widdowson would be expected to be—"Yes, fortification of wheatmeal flour with calcium salts would overcome the adverse effects of its phytate on calcium absorption so long as there was some vitamin D either in the diet or in the body, but not otherwise."

The answer to question 2 (b)—Will vitamin D restore the calcium absorption and balances of a brown bread diet to their white bread levels?—could, also have been foretold on the information recorded in Chapter XIV. Since the subjects studied by McCance and Widdowson had been receiving an ordinary, well balanced diet before the experiment was begun, it would be expected that they would have considerable body reserves of vitamin D and that the effect of adding more of the vitamin to the food would be small or even absent. As shown above in the case of puppies on a diet completely free from vitamin D, it usually took several weeks or, if large quantities of the vitamin had been previously given, even two or three months, to eliminate all the vitamin from the body. This point as regards human beings will be discussed later in the chapter.

It was seen in Experiment 20 (Chapter XIV) that the

interaction of vitamin D, calcium, phosphate and phytate was delicately balanced and that an increase in vitamin D above a certain quantity did not, under the experimental conditions tested, raise the calcium retention, while an increase in the dietary Ca might at some stages be more effective in this respect than an increase in the vitamin D. Thus in puppies the calcifying qualities of a diet containing 5 i.u. of vitamin D_2 and an extra 0.5 g. of Ca were equivalent to those of a diet containing 100 i.u. of vitamin D_2 without the additional Ca. No doubt the same applies to human beings, so that increasing the vitamin D intake above a certain level, which may be low as in the case of dogs, would not be expected to bring about a further increase in the retention of Ca or, to put the same fact in other words, further to increase the antagonism to phytate.

The actual answers obtained by McCance and Widdowson to the questions set out above are as follows:

"(a) The calcium, magnesium, phosphorus and potassium in diets made up with 92% flour were less completely absorbed than the same minerals in diets made up with 69% flour. Hence in defining calcium requirements it is essential to state the nature of the cereal in the diets.

(b) Sodium phytate added to 69% flour depressed the absorption of calcium and magnesium, but not of potassium. About 50% of the phosphorus in sodium phytate was absorbed.

(c) Vitamin D did not materially improve the absorption of calcium from diets made up with 92% flour.

(d) Fortifying the bread with calcium salts improved the absorptions of calcium, and prevented a loss of calcium from the body if this had been taking place. The carbonate and phosphate were equally efficacious. The addition of calcium carbonate slightly depressed the absorption of phosphorus."

The demonstration by McCance and Widdowson

(1942a) of the interfering effect of phytate on calcium absorption from the alimentary canal in human beings is sufficiently important to warrant a fuller account of this particular test. In the preparation of the bread, sodium bicarbonate and potassium hydrogen tartrate were used as leavening agents, and 50 cc. (0.55 g. of phytate P) of a solution of sodium phytate were incorporated in each 1 lb. loaf. The bread so prepared contained about 130 mg. of phytate phosphorus per 100 g. This was rather more than the brown bread previously used, for which the corresponding figure was about 100 mg. In these tests calcium balances were obtained for each of 8 people: (a) when white bread was eaten and (b) when white bread containing the additional sodium phytate was eaten. The results shown in Table 38 were obtained.

TABLE 38

Subject	White bread				White bread and sodium phytate			
	Period of observation, days	Calcium			Period of observation, days	Calcium		
		Intake, mg / day	Absorption mg / day	Excreted in urine, mg / day		Intake, mg / day	Absorption, mg / day	Excreted in urine, mg / day
E. B. .	21	500	307	254	21	453	107	155
N. K.. .	14	540	141	214	14	540	−111	128
R. M.. . .	14	645	36	97	21	630	−105	46
P. S...... . .	14	578	216	216	21	597	−9	168
B. A... ...	21	510	63	109	21	436	−11	57
R. W.. .. .	14	525	157	139	21	432	13	64
A. M. .. .	21	416	127	122	21	518	6	86
E. W..... .	14	440	41	84	21	476	−46	46

These figures show how the absorptions from the alimentary canal and urinary excretion of calcium were

changed when sodium phytate was added to white bread. All the subjects reacted in the same way. Their urinary calcium fell, their absorption of Ca went down so much that a number of subjects showed a large negative alimentary balance, for there was more calcium in the faeces than in the food under the influence of sodium phytate. McCance and Widdowson concluded that the addition of sodium phytate to white bread reproduced in an exaggerated degree the effect of brown bread on calcium metabolism.

The above experiment might be criticised in that sodium phosphate containing the same amount of phosphorus as in the sodium phytate was not added to the white bread; but it has been shown above that, in dogs (Experiments 11, 13, 14, 15 and 16), when sodium phytate is matched against sodium phosphate containing an equal quantity of phosphorus, phytate has a specific effect in reducing calcium absorption from the gut as compared with phosphate, when vitamin D is present in the body or in the food.

A better answer was supplied by McCance and Widdowson (1942 b) when they proceeded to do a more closely controlled human experiment, by comparing the effect on calcium absorption of flour containing phytate with that of other flour, the phytate of which had been converted to phosphate. The experiments were made on 6 subjects, 3 men and 3 women. There were four tests made on each person, only 3 of which will be mentioned here —(1) a white bread control, (2) a brown bread control and (3) brown bread periods when the bread was dephytinised. In these experiments the white flour was one of very low extraction (figure not given); the brown bread was made of 83 parts of white flour and 17 parts of bran. The brown bread in which the phytate had largely been hydrolysed by the phytase of the bran was comparable

in composition to the brown bread of the control, except that the phosphorus was in the form of inorganic phosphate and not as phytate. The typical results shown in Tables 39 and 40 were obtained on 2 of the 6 subjects.

TABLE 39

The intakes and absorptions of calcium

Type of bread	Subject 1			Subject 2		
	Intake, mg / day	Absorption, mg / day	Absorption, % of intake	Intake mg / day	Absorption, mg / day	Absorption, % of intake
Brown	550	89	16	522	57	11
Dephytinised brown	590	231	39	566	169	30
White	488	250	51	478	219	46

TABLE 40

The intakes and absorptions of phosphorus

Type of bread	Subject 1			Subject 2		
	Intake, mg / day	Absorption, mg / day	Absorption, % of intake	Intake, mg / day	Absorption, mg / day	Absorption, % of intake
Brown	1940	900	46	1650	819	50
Dephytinised brown	1900	1230	65	1550	980	63
White	1260	923	73	1090	875	80

It will be seen from the results that the calcium absorption in these two subjects was less when the bread was made from untreated brown flour (phytate intact) than when it was made from dephytinised brown flour (phytate hydrolysed to phosphate). The calcium absorption was highest in the white bread period when there was no phytate and only inorganic P in the food. As regards phosphorus absorption, this was also lowest in the brown bread control period, when there was much phytate

present, and greatly increased in the dephytinised bread period.

These results again confirm, in the case of man, those obtained on dogs and show that brown flour has a greater anti-calcifying action than white flour: they also indicate that it is largely due to the phytate content of the former. Further, it is evident that phosphate interferes to some extent with the absorption of calcium, but that this effect is much less pronounced than that of phytate.

Another interesting investigation of phytate metabolism in human beings was made by Cruickshank, Duckworth, Kosterlitz and Warnock (1945), who studied the effect of increasing the calcium intake of flour in adult subjects on the digestibility of phytate in a diet rich in oatmeal (165 g. daily). The word "digestibility" in this publication indicated the disappearance of substances from the ingesta during the passage through the intestine, irrespective of whether it was due to the subject's own digestive action or that of the intestinal flora. Unlike McCance and Widdowson, who found about 50% of the phytate P of wheat flour excreted in the faeces, these investigators found that most of the phytate P (94%) disappeared from the gut and, at the same time the phosphorus absorption was normal. In previous chapters it has been seen that the two known circumstances which tend to increase phytate disappearance from the gut in dogs are (1) the presence of vitamin D in the body, and (2) a low calcium intake. Of these two factors, there was certainly some vitamin D in the food and probably substantial stores in the body in the four subjects of the human experiment. The question is, was their calcium intake really low? The authors state that it "approached requirement" when the average daily intake reached 582 mg. daily. When it was increased from 582 to 765 mg. *this digestibility of the phytate was reduced only from*

94% to 92% of the intake. When, however, the calcium intake was raised from 765 to 1076 mg., the digestibility of phytate was reduced from 92% to 77%. There would probably be some criticism of the statement made by the authors that the calcium intake "approached requirement" when it was at a level of 582 mg. daily, considering that the diet at the time contained 574 mg. of phytate P out of a total phosphorus intake of 1972 mg. This criticism would be supported by the fact that, under these conditions, the average daily loss of calcium of each of the four subjects under experiment was 90 mg. This may seem a trivial daily loss, but it means that, in the course of one year, the average loss of each of these subjects would have been 33 g. of calcium and, extended over a series of years, would undoubtedly have brought about an undesirable condition of osteoporosis and weakening of the bones.

Cruickshank et al. (1943) also quote in corroboration of their results the work of Steggerda and Mitchell (1939) to show that a high oatmeal intake did not interfere with Ca absorption. It may be worthy of remark, especially for British readers, that when Americans speak about "oatmeal" they usually mean "oatmeal porridge," and therefore it is most likely that the 224 g. of "oatmeal" recorded by Steggerda and Mitchell as having been eaten at breakfast really represents the amount of oatmeal porridge consumed, i.e. between 30 and 50 g. of oatmeal, and not 224 g., which appears to be a very large breakfast.

There are some further interesting points about this work (Cruickshank et al.) on human beings. For instance, it became clear in the puppy experiments (Chapter XIV) that the influence of dietary calcium on phytate depended largely on a direct interaction of these two substances in the intestine. Some details of the work under discussion suggest that similar conditions hold in the human being. Practically all the phytate in the diet was taken as oatmeal

cake and not as oatmeal porridge. The inclusion of oatcake allowed a dissociation in the ingestion of phytate and calcium which oatmeal porridge would probably not have allowed, since it is usual to take porridge with milk. One subject of this work, D.T., took most of her oatcake and all her milk at breakfast and it was this case that showed the greatest decrease in digestibility of phytate from 94% to 66% on raising the calcium intake from 515 mg. to 1038 mg. On the other hand, another subject, W.S., ate all his oatcakes at breakfast and drank all his milk at 10 p.m. Obviously in this case the phytate of the oatcake and the calcium of the milk had no opportunity to combine, with the result that the digestibility of the phytate remained at a level of 86% to 88% when 614, 792 and 1099 mg. Ca respectively were ingested daily. It must be a point of some significance to those carrying out feeding experiments in the future to realise that, in work of this kind, the result depends not only upon what food is given but when it is given, and that, in a case such as is under discussion, calcium and phytate may on one occasion react on each other's metabolic changes and on another have no direct effect on one another. (Experiments 23–25 demonstrated that this question of relative times of ingestion probably also applies to vitamin D supplements). It might be inferred from this Aberdeen work that a high cereal diet containing much phytate does not call for a correspondingly high calcium intake. This would, however, be an unwise deduction, for nothing is more certain than that a high cereal content of the diet, involving a large amount of phytate, calls for a correspondingly high intake of calcium as well as sufficient vitamin D, especially in the young and during growth; otherwise both bone and tooth formation will be defective and growth will be suboptimal. In the case of the adult, the bones will be depleted of their calcium owing to a long-

continued negative balance and osteoporosis may well develop, with easily fractured bones. As was seen in Chapter XI, in dogs this may eventually lead to osteomalacia when vitamin D is also deficient.

The results of the experiments of Hoff-Jørgensen, Andersen, Begtrup and Nielsen (1946), in which they gave to infants quantities of sodium phytate sufficient to combine with about 50, 75 and 100 per cent. respectively of the total calcium of the diet, are also of interest. They found that a large decrease in the calcium absorption followed these additions and, at the same time, an increase in phosphorus absorption. Positive Ca balances were either greatly reduced or made negative. These results were in general accord with those of McCance and Widdowson on adults described above and, of course, with the dog experiments. In some of the tests reported by Hoff-Jørgensen et al. the amount of phosphorus absorbed was greater than the amount of non-phytate phosphorus in the diet. It seems that some of the phosphate which was split from the phytate in the intestine (about 35% of the intake) must therefore have been absorbed. This result is also similar to one described in dogs in Chapter XV (Experiment 16), where there was evidence that phytate P had been absorbed and excreted in the urine as inorganic phosphate. The above investigators, again like McCance and Widdowson, found that a supplement of vitamin D_2 had but little effect on the inhibitory action of phytate on calcium absorption; in this case they gave 100 mg. of ascorbic acid and 600 instead of 2,000 i.u. of vitamin D_2. Further remarks on this point are made below.

Hoff-Jørgensen, Andersen and Nielson (1946) made further experiments on two boys aged ten years. During three periods of five days these boys received a diet poor in phytate; one rich in phytate was then given for three similar periods and finally the first diet was repeated. The

diets contained 500 g. of milk and about 0.9 g. calcium daily. The effect of the phytate was to cause a great reduction in the absorption and retention of calcium and an increase in the absorption and retention of phosphorus. The authors suggest that, if the children had been kept on the diet rich in phytate for a longer period, their Ca absorption might gradually have increased, for it was considerably lower during the first five days of this diet than in the following period. In both subjects, however, the increase in Ca absorption did not seem to continue beyond the second phytate period. Hence Hoff-Jørgensen and his colleagues suggest that their results indicate that in these subjects the "adaptation" to conditions less favourable for calcium absorption was already complete in seven days. The problem of adaptation of the digestive processes in such a way that increasing quantities of phytate can be hydrolysed to phosphate, when the diet is constant over a period of time, may obviously be one of important physiological significance and deserves further study.

On page 398 a reference was made to the fact that, when wheatmeal flour was eaten, McCance and Widdowson were unable to increase the calcium absorption of their experimental subjects to the white bread level by adding vitamin D (2000 i.u.) to the diet. Similarly, it was mentioned above that Hoff-Jørgensen, Andersen, Begtrup and Nielsen were unable to alter the inhibitory action of phytate on calcium absorption by a daily supplement of 600 i.u. of vitamin D_2. These results are almost certainly due to the fact that the subjects already had substantial stores of vitamin D_2 in their bodies and probably the basal diet also contained a certain amount of the vitamin. Nothing has been more clear in the experimental work above described than the fact that the reserves of vitamin D in the body are very effective in promoting

Ca absorption and that, so long as they are present, additional vitamin D in the diet makes but little difference. Many people do not realise how long the stores of Vitamin D_2, even in normal people, can serve the function of this substance in the animal economy and the time it may take to rid the body of the last traces. In their work on osteomalacia in China, Hannon, Liu, Chu, Wang, Chen and Chou (1934) had an opportunity of investigating a patient suffering from this disease, who was devoid of all vitamin D reserves. They administered irradiated ergosterol over a period of 16 days in what they say was a small dosage and then examined the calcium balance for several months. They found that the beneficial effect of this small amount of vitamin D_2 on calcium and phosphorus metabolism continued for at least 4 months, and perhaps longer, after administration of the vitamin had ceased. It is doubtful whether, during this period, additional vitamin D_2 would have had any effect on calcium and phosphorus retention. Other work by Liu and his colleagues (1940) in China also pointed to the ineffectiveness of additional dietary vitamin D when the reserves of the vitamin were good and to its great effect on calcium retention when there were no such reserves in the body. These results were obtained on two lactating women suffering from osteomalacia, one of whom had been dosed with vitamin D just prior to the experimental work, while the other had had no such treatment. Thus, the woman with reserves was in a condition where her osteomalacia was healing, whereas in the second woman the disease was active. When vitamin D was added to the diet of both it produced little change in the Ca and P balances of the first patient, but in the second case it not only caused rapid improvement in the condition, but also a significant increase in the calcium and phosphorus retention.

A warning may be introduced here. While it is clear

that the absence of effect of additional supplies of vitamin D on Ca retention in the human experiments referred to above is due to the fact that the body contains reserves of this vitamin, it is also important to remember the limited value of estimations of Ca retention as a measure of calcification processes. This subject is discussed at length in chapter XIV and it is shown that, although small quantities of vitamin D may promote optimal absorption of Ca from the intestine, more of this vitamin is required to produce bones of the highest calcification, because it also influences bone structure. In other words, other conditions being equal, more vitamin D is required to produce optimal bone formation than is required to promote a maximum Ca absorption. This fact has not been shown to apply to human beings but, if it does, as there is every reason to believe is the case, Ca balance tests may be as limited in their significance, when applied to children, as they are in growing animals.

A number of other papers on phytate in relation to rickets and osteomalacia in China have been published and, while many are full of interesting facts, the dietetic and therapeutic conditions are often so complicated that they do not lend themselves to detailed description and discussion. It is clear, however, from all this work that cereals, because of their phytate content, must play a large part in the development of osteomalacia in China and that it is not simply a disease due to absence of vitamin D. In many districts in the East it seems as if the diet had all the qualities for bringing about most intense decalcification of bones. Thus, diets containing little or no vitamin D and with a high phytate and a minimal calcium content are by no means uncommon, and it is this combination of dietary abnormalities which brings about the extraordinary and intense degree of osteomalacia that develops in those countries.

Surprise has sometimes been expressed that, although the diets of Eastern races, as in India and China, are very defective in calcifying qualities, the large amount of sunshine has not prevented the severe osteomalacia that is often found among their inhabitants. Sunshine acting directly on the skin certainly does bring about an increase in calcium retention in severe cases of osteomalacia in these countries, as shown by Chu, Yu, Chang and Liu (1939), and it must therefore be accepted that cases of this disease have not been exposed to sunshine. When once such a disabling disease develops, it is almost impossible for the patients to move and they probably lie about in their dwelling places.

Enough has been written in this chapter to show that the anticalcifying effect of cereals, whose action on animals has been described in detail in previous chapters, also exerts a similar action on man which, in children, may result in rickets and hypoplastic teeth and, in adults, may produce osteoporosis and osteomalacia. Since the main facts seem to hold, it is likely that the more detailed effects, as described in the animal experiments, also hold in the case of man. It is also obvious that the subject is one of great importance to human well-being and that, in any endeavour to meet the nutritional needs of individuals and communities, the facts described must receive due consideration.

Chapter XVII

CEREALS AND INTERFERENCE WITH CALCIFICATION

Summing Up of Results above Described

The object of the investigation described in Part II of this book (corresponding to the 4th and 5th of the five lectures given) was to study the rickets-producing effect of cereals, a phenomenon shown experimentally in 1919 (see p. 208). In Chapter XI a closer consideration of the problem seemed to reveal that the action of cereals depended on two properties, (1) a growth-promoting effect which is probably common to all cereals and (2) the presence of a chemical substance, plentiful in some, such as oatmeal, corn and wholemeal wheaten flour, and sparse in others, such as rice and white flour, which has the property of interfering with calcification of growing bones and teeth.

As regards the growth-promoting effect of cereals, no further work has been done and it is impossible to say how important it is from the point of view of bone calcification: more especially is it impossible to say whether cereals differ in any way among themselves in their power to promote growth. Here a distinction must be made between growth and increase in weight. For months at least, equal quantities of different cereals added to the same basal diet usually produce in litter-mates increases in weight of the same order, as can be seen in Figs. 43 and 51. A closer examination of the rates of growth of bones of litter-mates eating different cereals might show relatively greater differences than the weight curves but, if so, these differences are only slight and would not account for variations in the rickets-producing effect of the various

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ing problem to find out how it came about that some cereals with more Ca and P resulted in the formation of bones containing less of these elements than the bones produced by cereals containing smaller quantities of Ca and P.

It will be seen that the more recent experiments have consisted of close metabolic studies of the interaction of three substances which are themselves, or which contain, elements essential for calcium metabolism, namely, vitamin D, Ca and phytate, the last a rich source of P. If nature had supplied more phosphorus as inorganic phosphate and less in the form of phytate, the above described investigation would have been unnecessary. As it is, phytate provides much of the P essential to animals, and its unhappy property of combining with Ca in the intestine and so making this salt unavailable to the animal is the cause of much physical abnormality. It would be wrong, however, especially from a scientific angle, if all interest in phytate as a physiological study centred round its property of immobilising Ca in the gut. As a rich source of inositol, an essential dietary constituent in many animals, and a well recognised constituent of nervous and muscle tissue, phytate is clearly of some importance from another angle.

It is impossible to study the calcification of bones in growing animals without being impressed by the dominant position held by vitamin D. Take all vitamin D away from the food and ultimately, when the reserves of this substance are lost, the animal cannot retain sufficient Ca, however much is added to the food. This point has been dealt with in Chapter XIV and no further emphasis will be given here, except to repeat that, when what appears to be a relatively small amount of this vitamin is supplied to the body, further additions do not increase the powers of the body to absorb and retain Ca. This

cereals. Certain it is that rickets would not be produced in young animals by diets which did not allow bone growth, so that it is possible that further investigation of the growth-promoting action of cereals might open up a new field. As pointed out in Chapter XI, too little is known about these physiological effects of cereals, especially about that portion of their chemical make-up upon which growth is dependent.

The second characteristic of cereals which brings them into association with bone calcification has been closely examined and discussed in Part II of these lectures. This is the presence in the cereals of a substance having powerful anticalcifying properties which, by interfering with calcium metabolism when there is a deficiency of vitamin D, causes the production of rickets in young growing animals and, in adult animals, a decalcifying effect which may lead to osteomalacia. This rachitogenic substance proved to be phytic acid or inositol hexaphosphoric acid, a compound which has long been known to exist in cereal grains and, indeed, in all seeds in the form of the insoluble Ca and Mg salt—phytin. Phytin itself is not rickets-producing but, as shown above, oats and almost certainly other grains contain phytic acid which is not wholly satisfied by Ca and it is this form which has the toxic anticalcifying action. It was found early in the work that the phytate action could be largely antagonised by the addition of extra calcium to the diet, especially in the first weeks of the experiment, while the presence of vitamin D completely prevented rickets, although as shown later, it did not necessarily prevent the predatory effect of phytate on calcium. Since different cereals contain different amounts of salts of phytic acid, it soon became apparent that the rickets-producing effect of cereals was related not only to their phytate content but also to their different Ca contents. Certainly it was an interest-

one point of view this was the focussing point of the whole investigation, since the object was to determine the substance in cereals responsible for their rachitogenic effect, and phytate proved to be this substance. This fact having been established, it became easy to understand the basis of the earlier observations made before there was any idea of the nature of the substance, namely, that this rickets-producing effect of cereals was reduced both by boiling them with mineral acid and by subjecting them to a malting process, for it was known that phytate could be hydrolysed by both forms of treatment. A full account of this part of the work, which led to the elucidation of the *conditions under which* phytate and hydrolysed phytate (inorganic phosphate) interfered with calcium absorption from the gut, is given in Chapter XIV. In the presence of vitamin D, phytate specifically depresses calcium absorption while phosphate of the same P content has hardly any effect. Only when the vitamin D reserves are used up and there is none in the food, do phytate and phosphate act similarly as anticalcifying agents, although even under these conditions it is probable that phytate is the more powerful of the two in this respect. The specificity of action of phytate in depressing calcium absorption and retention in the presence of vitamin D has important implications, both practical and theoretical. From the latter point of view it may be claimed that it is the effective answer to the Council on Food of the U. S. A. who reported in 1937 that 'there is no good evidence of a decalcifying factor in cereals and that the hypothesis of the evidence of such a factor is not needed to explain experimental results. . . . Phytin P may or may not be completely available to the organism, depending on the extent to which it is hydrolysed by intestinal bacteria. The experimental results observed and reported in the literature may be explained on the basis of the Ca and P

does not mean that the additions have no effect on the bones, for it was shown in Chapter XIV that increasing the vitamin intake resulted in an improvement in bone quality even when it had no effect on calcium absorption. It does this by controlling the proportion of organic to inorganic substances. Attention has been given in this work to the action of vitamin D in stimulating the absorption of Ca from and hydrolysis of phytate in the gut, but it seems certain that this does not explain all the action of the vitamin, and that it also plays an important part in promoting the deposition of a calcium-phosphorus compound in the osteoid tissue of growing bone and in producing perfect bone structure.

Just as vitamin D holds a dominant position in calcification up to the point when the supplies of this substance in the body are sufficient for maximum effect, so also are those other factors in calcification, namely Ca and phytate P, of prime importance to the welfare of the body at certain times and under certain conditions, while at other times they may be of relatively less significance. When it was substantiated that phytate was the offending anticalcifying substance in cereals, it became the main object of the present work to study the interactions of these three substances in the hope of finding the particular conditions under which each factor in turn assumed greater importance than the other two. It will be obvious from the foregoing account that this subject is one of great complexity and, although the tangled skein has to some extent been unravelled, it is certain that much remains to be done even in this respect. It will also be seen that some fundamental knowledge in physiology will be gained when the action of these substances in the body is fully understood.

Let us now review the part played by phytate in calcium metabolism as revealed by the foregoing work. From

dent when the vitamin D reserves run out and the diets of respective litter-mates contain phytate and inorganic phosphate of the same P content. It will be noticed in some of the graphs representing Ca absorption and retention under these conditions that the curve of an animal receiving phytate descends, indicating loss of power to absorb Ca from the gut, several weeks before the curve of a litter-mate receiving phosphate shows a similar reaction. This change takes place sufficiently often to suggest that phytate disperses vitamin D reserves in the body more quickly than phosphate. It may indicate that under normal conditions a diet rich in phytate not only needs a larger Ca supply to make up for the Ca carried away by the phytate in the faeces, but also that it makes rather bigger demands on vitamin D, both in the body and in the food, than does a diet in which the P is present in the form of inorganic phosphate.

While phytate antagonises vitamin D in the sense that the latter ensures Ca absorption from the gut while the former reduces it, vitamin D is not passive towards phytate, but endeavours with some success to prevent its action by increasing the hydrolysis of the phytate to inorganic phosphate. By so doing it also indirectly increases the amount of Ca absorbed from the gut. Indeed it has been shown in Chapters XIV and XV how similar is the action of vitamin D in controlling Ca absorption and the disappearance of phytate from the gut. Both actions are dependent on relatively small quantities of vitamin D in the body and are not increased by further supplies; both are controlled by body reserves of this vitamin when there is none in the diet. It is possible that, if we knew how vitamin D brings about the destruction of phytate, we would also know how it induces Ca absorption.

Chapter XV was devoted to a consideration of the method whereby vitamin D causes the hydrolysis of phy-

ratio in the diet together with the knowledge of the availability of the P'. Some of these statements have been dealt with in the foregoing chapters. Here it will suffice to say that phytate in cereals fulfils all the conditions of a specific decalcifying factor.

The diminished absorption of Ca in the presence of phytate was established primarily by the production of rachitic bones of lower Ca content in young animals by diets which allowed the vitamin D reserves to be gradually lost. Later the method of balance experiments made it clear that less Ca was absorbed and retained by the body in the presence of phytate. But probably the most effective way of demonstrating this relationship was by determining the unabsorbed Ca in the faeces under these conditions. Curves showing the quantitative connection between unabsorbed Ca and phytate in the faeces and how these are related to the phytate intake were shown in Chapter XIV (Fig. 58). Here it was seen how increasing the food phytate, in the presence of vitamin D, increased the unabsorbed phytate and how this, in turn, led to an increase in faecal Ca and so reduced the amount available to the animal. It is most important that it should be realised how powerful this anticalcifying effect of phytate can be in the presence of vitamin D. When the diet is rich in phytate, optimal bone calcification cannot be assured simply by giving an adequate supply of vitamin D. It is true that the latter will prevent all rickets, but, in order that under these conditions, the bones may be properly calcified rather than osteoporotic the intake of Ca must also be large.

Although the evidence is not conclusive, there are indications in some of the experiments of another relationship between phytate and vitamin D which may prove to be of significance. It seems to be another aspect of the fight between phytate and vitamin D and becomes evi-

dietary vitamin D, in the absence of body reserves, may be sub-optimal in its effects as regards Ca retention, but become much more powerful in this respect when a little Ca is added to the food. It is at that time that calcium comes into its own and holds the balance between a diet which is beneficial and one which is harmful from this point of view. Again, when the diet is rich in phytate, calcium assumes another important rôle, because it has been seen above that, even in the presence of vitamin D, phytate removes calcium from the gut and makes it unavailable. Such deprivation may result in the body not getting enough calcium even though the supplies seem good and the vitamin D adequate. Under these conditions much more Ca is needed in the food. This action has been discussed above (p. 349), where it was shown that increasing the dietary calcium compensated for that precipitated by the phytate in the faeces. Thus here are two occasions when the Ca of the diet becomes of crucial importance for the attainment of optimum calcification: (1) when the vitamin D supplies are minimal, for at this time vitamin D is much more effective if the Ca supplies are increased, and (2) when the phytate intake is high, for, owing to the precipitation of Ca in the gut, so that it is unavailable to the body, it is essential that the Ca intake be large.

Having now set out some of the main interactions of these nutritional substances concerned with calcification, and having discussed, in particular, the conditions under which each factor seems to hold a dominant position, it might be well to consider briefly the practical significance of this chemical function in terms of the physical life of man and animal. Clearly the object must be to procure conditions for optimal retention and deposition of calcium. In the growing animal this would result in well formed bones and teeth. So far as the bones are concerned, there should be an increase in size and in strength, with a greater

tate to inorganic phosphate. The greater effectiveness of small quantities of vitamin D when *injected intravenously* before food, as compared with equal amounts *given by mouth* before food, indicates that the vitamin effect is at least partly humoral in its action and not a direct action on the phytate in the gut. Evidence is also adduced in favour of the view that the intermediary agent in the hydrolysis is an enzyme, phytase, which is present in the gut even at the duodenal end of the small intestine. It is suggested, on indirect evidence, that vitamin D in the bloodstream might cause the development of phytase in the gut just as, although probably not by the same mechanism, secretin causes a secretion of pancreatic juice. This however, is surmise at the present stage. Indeed, no clear proof has yet been obtained that vitamin D increases the phytase in the contents of the alimentary canal, although the evidence that a phytase is naturally present there and that the presence of vitamin D in the diet causes an increased hydrolysis of phytate is strong. It may well be that the increase in phytase activity caused by vitamin D is not due to a larger secretion of phytase but only to the production of better conditions for this enzymic change such as less Ca and a more optimal pH produced by the vitamin. The whole problem is at a most interesting stage. It may also be added that no support has been obtained in this work for the suggestion, so often made by past investigators, that the hydrolysis of phytate in the gut is due to bacterial action. Almost certainly it is the result of a naturally occurring and naturally secreted phytase.

Let us now consider the question of calcium itself, and see whether there are occasions when this substance assumes a more dominant position than usual. When there are no reserves of vitamin D, it has been seen that all or most of the calcium in the diet is excreted, no matter how large the intake. On the other hand, a small quantity of

contains but little phytate. In the Western Hemisphere the consumption of phytate was greatly reduced before the war in this country, and both before and since the war in the U. S. A., by removing, in the milling process, the phytate-rich bran and germ from the wheaten grain and so preparing a white flour of low extraction composed almost entirely of endosperm relatively poor in phytate. Then, again, the method of preparing and cooking cereals may effect a further reduction in the phytate content of the diet by the conversion of the phytate to inositol and phosphate by phytase. The phytate hydrolysis in the baking of bread was described in Chapter XIII. Another method largely practised, in which the hydrolysis of phytate occurs, is that of malting and fermentation, so that both in malted grains and in malt extracts, also in alcoholic drinks, the original phytate has largely or entirely disappeared. (This subject is also discussed in Chapter XIII.) This method of food preparation involving the hydrolysis of phytate is also of great importance in tropical countries where a valuable article of dietary is the native beer made from corn (maize). It will be seen, therefore, that there are few communities in the world that have not yet learned by instinctive means methods either for totally excluding the phytate from the cereal eaten or for hydrolysing it to inositol and phosphate, and thereby reducing its possible anticalcifying action.

The question arises—is it the better practice to remove the phytate of cereals from, or to include it in, the diet? Phytate when hydrolysed produces two substances which are essential for life, namely, inositol and phosphate, and it would therefore seem better if the diet should be rich in either phosphate produced from hydrolysed phytate or in phytate itself, so long as most of the latter is hydrolysed in the gut and absorbed from the alimentary canal, thereby losing its power to deprive the body of calcium, either

resistance to fracture and to other deformity. In the case of the teeth it would also mean, as shown by M. Mellanby, better growth of the jaws, improved spacing of the teeth and a reduction in the incidence of caries. These are certain results of better calcification, but there may be others. For instance, improved calcium metabolism may mean also better muscular development and strength because many of the nutritional conditions which affect bone undoubtedly affect muscle. Also, since defective calcium metabolism produces in young animals lethargy, and even inactivity, to such an extent as to border on paralysis, improvement of calcium metabolism might be accompanied by changes which would allow increased activity and alertness. However, some signs of defective calcium metabolism in man, such as those of bones and teeth, are only too well known and there are probably others at present unknown, such as resistance to some forms of infection and possibly other diseases, which will be revealed by their reduction or disappearance when nutritional needs related to calcium metabolism are made optimal.

It may be asked whether, in view of the action of cereals with high phytate content, they are in any way a menace to perfect calcium metabolism and the answer is, as seen above, that they need not be, if the diet contains sufficient calcium and vitamin D. Of these two food factors, the present work has laid particular emphasis on the need for a high dietary intake of the first, namely, calcium, when the diet is rich in phytate.

It is a matter of interest to see how instinctively, and with no knowledge of the subject, different communities have learned to deal with the cereal moiety of the diet in so far as phytate is concerned. Many peoples, especially in the Eastern Hemisphere, although consuming cereals on a large scale, have avoided the difficulty by choosing as their staple article of diet a cereal such as rice, which

rickets has disappeared and, as will be seen below, the teeth of the younger generation are showing improvement.

Modern experimental studies of calcium metabolism, including those described in the foregoing chapters, have not only provided the scientific foundation for improving the health and physique of the general population, but they have also emphasised the urgent need for the incorporation of these doctrines into human dietetics. Fortunately for Great Britain, most of these nutritional methods for improving calcification have now been adopted in some degree, mainly as the result of Government action during the recent war. In course of time the methods will no doubt improve, with even better results. While it would be untrue to say that all the changes in dietary made before and during the war have aimed at the improvement of calcium metabolism of the individual, it is certainly true that practically all the steps taken have in one way or another had this effect. For instance, the greater consumption of milk in childhood has undoubtedly brought many nutritional advantages, but of prime importance is its vitamin D and large calcium content. Even prior to the war, this development had been well advanced and arrangements had been made for the provision of cheap milk to school children. With the outbreak of the war the whole feeding arrangements of the country, including those for the distribution and assignment of milk, came more and more under the direct control of the Government, largely because the restrictions in imports made it essential that the food available should be distributed to the best general nutritional advantage.

One consequence of this was the adoption of a policy for the provision, among other things, of free or cheap milk and cod-liver oil for all children up to the age of five and important priority provision of these foods to pregnant and nursing women. This policy ensured an increase

from the food or from the tissues. There can be no doubt that a diet rich in high phytate-containing cereals, such as oatmeal, corn or whole meal flour, can be compatible with good calcification and all the benefit this brings, if at the same time it is associated with foodstuffs rich in calcium and vitamin D. One of the best instances of the development of a natural taste for a diet of this kind is that of oatmeal porridge eaten with a large amount of milk—a mixture which at one time formed a substantial part of the Scottish diet (see page 213). In this case the oatmeal supplies the phytate, while the calcium and vitamin D of the milk ensure that its possible anticalcifying effects are neutralized and some or much of the products of its hydrolysis are retained for the use of the body.

It is certain that these nutritional biochemical reactions *which dominate physiological calcification are most important* from the point of view, not only of health and physique, but also of life itself, and any community whose main supply of food is cereal, especially if rich in phytate, *must learn instinctively* or *by other means how such food* should be eaten and in what combinations, in order to keep in check its anticalcifying action; otherwise it is certain that the community will become physically degenerate. The poor calcifying quality of the diet largely made up of bread not balanced by foods rich in Ca, such as milk and other dairy products, was undoubtedly responsible for the poor physical condition of many people in Great Britain in the latter part of the last century and at the beginning of the present century. A large proportion of the child life of Britain was rachitic at some stage of growth and bone deformity was present in many of the adults as the result of this early disease; the teeth were deplorable, and the average size of the population was undoubtedly suboptimal. With the change of feeding habits, the physique of the population has been transformed;

the extraction rate of the flour being raised from the pre-war figure of 70-73 per cent. up to 85 or even 90 per cent. of the whole wheaten grain during the war period. Although the increase in extraction rate of flour has been advocated strongly by those with knowledge of nutrition as a beneficial procedure in itself, it is doubtful whether it would have been adopted as part of the Government feeding policy in Britain, had not the blockade and later the economic situation forced the adoption of this measure. The use of a national flour of, for instance, 85 per cent. extraction increased the phytate P content of the flour greatly, i.e. from about 0.05 per cent. to about 0.12 per cent., and, as shown in the foregoing chapters, it was this addition of phytate which further increased the need for adding extra calcium to the flour. This instance of mass-control of dietary by the addition of calcium carbonate to the National wheatmeal flour is probably one of the main nutritional changes responsible for the improvements in health and physique seen in Britain during recent years.

In passing, it may be noted that the policies of the U. S. A. and Britain as regards bread since early in the war have been quite different. In the U. S. A. white bread of low extraction flour has continued to be eaten and some of the vitamins of the grain lost in milling, including aneurin (thiamine), nicotinamide and riboflavine, have been restored to the flour, whereas in Britain the vitamins of the grain have been largely retained by the use of a high extraction flour and the increased phytate content neutralized by adding calcium carbonate in the quantities stated. It may be that the American diet generally is so good, as the result of the high standard of living, that the addition of vitamins to the white bread has had little or no effect on the general health in that country. In Britain,

in the calcium and vitamin D intake of the poorer part of the population, especially during that period of life when the need was greatest. This large increase in consumption of milk by priority classes, which amounted to more than 50 per cent. of the whole milk production, left the rest of the adult population of the country with small supplies, a fact which was and still is well seen by the allocations to the non-priority classes. These have often been reduced over long periods to an allowance of two pints weekly, or rather less than ¼ pint (150 ml.) daily. Deprivations of this nature, which have not been helped by the small amount of cheese and the very few eggs available, would probably have led to some disturbance of health in a large section of the British population, had not two steps been taken to counteract this limitation. These were (1) the addition of vitamins A and D to all margarine. Since the allowance of margarine per individual has been, throughout the rationing period, of the order of three to four ounces per week, each person has been supplied daily through this source alone with 12 to 50 i.u. of vitamin D and approximately 200 to 300 i.u. of vitamin A; and (2) the addition to all flour of calcium in the form of Creta preparata since May 1942. On that date 7 oz. of calcium carbonate per 280 lb. of flour were provided and from August 1946 to the present day this has been raised to 14 oz. per sack. Thus, in flour alone during the bread rationing period, each person on the average has been provided with a possible daily supply of 300 mg. Ca, equivalent to the Ca of about 250 ml. (½ pint) of milk. Quite apart from the diminished supplies of calcium resulting from the greatly restricted quantities of dairy produce available during and since the war, and the need therefore of raising the calcium intake in some other way, the matter became one of even greater urgency owing to

and a diminished susceptibility to dental decay. In order to obtain evidence on these points, May Mellanby, with the assistance of a number of colleagues, has made a series of investigations of the teeth of children aged five years attending schools under the authority of the London County Council. The first inspections were made in 1929 and were published by the Board of Education, 1931, and similar surveys in the same schools were made in 1943, 1945 and 1947 (Mellanby, M. and Coumoulos, 1944, 1946: Mellanby, M. and Mellanby, H., 1948). Some of the main results are shown in Fig. 73. It will be seen that in 1929 under 5 per cent. of the children examined were caries-free or nearly caries-free. Between 1943 and 1945 the figure increased from 24 to 28 per cent., and by 1947 it had reached 38 per cent.

Detailed results of the 1929 survey are not now available, but in the three more recent surveys the assessment of each tooth of every child for both structure and the incidence and extent of caries indicated that there was a progressive improvement in all respects from 1943 to 1947. The proportion of teeth of good structure increased from 31 per cent. in the first of the three surveys to 38 per cent in 1945 and to 47 per cent in 1947. The increases in teeth free from caries were from 70 per cent in 1943 to 74 per cent in 1945 and to 80 per cent in 1947; in other words, between the first and the third of these war-time and post-war inspections the proportion of carious teeth in 5-year-old children attending these London schools diminished by 33 per cent.

It is of interest to note that a similar survey of children of the same age group in residential institutions in 1945 revealed the fact that the percentage of such children free from caries was very much higher than that in London County Council school children, who for the most part lived in their own homes. There were 66 per cent of

on the other hand, the addition of dietary supplements to flour and margarine has undoubtedly been a public health policy of major importance.

In spite, therefore, of the restrictions of food, especially as regards variety, in Britain during recent years, there is evidence that the general nutritional status of a large part of the population has been raised, partly because of the better distribution of food to the poor, partly because of the direction of protective foods essential for growth to the young and to mothers during pregnancy and lactation, and partly because of the mass control on the qualitative side of food composition. Just how much of the improvement is due to better calcium metabolism cannot be stated categorically, but most of the changes have had this aspect of nutrition as their basis and it is probable therefore that its effect has been of great importance.

One piece of evidence bearing on this question of the importance of normal calcification in human beings as a health problem will now be briefly mentioned, namely the great improvement in the dental condition of young people in Great Britain that has taken place in recent years. Since 1918, as the result of experimental work on animals and related clinical investigations on children, May Mellanby has not only described the nutritional conditions necessary during growth for the production of well-formed teeth, including the first account of the action of a calcifying vitamin on tooth structure, but she has also shown that the improvement in such structure in children is the first essential step in any plan to improve the state of the teeth, and especially to reduce the great incidence of dental decay.

If these results and this teaching were sound, it seemed probable that the recent dietary changes in Britain directed to improve calcium metabolism would be reflected in a raising of the standard of dental structure of children

and a diminished susceptibility to dental decay. In order to obtain evidence on these points, May Mellanby, with the assistance of a number of colleagues, has made a series of investigations of the teeth of children aged five years attending schools under the authority of the London County Council. The first inspections were made in 1929 and were published by the Board of Education, 1931, and similar surveys in the same schools were made in 1943, 1945 and 1947 (Mellanby, M. and Coumoulos, 1944, 1946: Mellanby, M. and Mellanby, H., 1948). Some of the main results are shown in Fig. 73. It will be seen that in 1929 under 5 per cent. of the children examined were caries-free or nearly caries-free. Between 1943 and 1945 the figure increased from 21 to 28 per cent., and by 1947 it had reached 38 per cent.

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these institutional children "caries-free" at that time, whereas among the London children examined only 28 per cent could be so classed (Coumoulos and Mellanby, M., 1947).

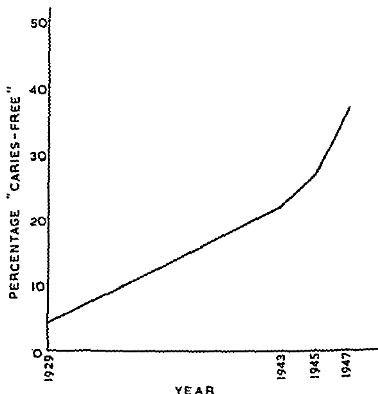


FIG. 73. Percentage of "caries-free" children aged 5 years in London County Council Schools in 1929, 1943, 1945 and 1947.
Note: Large increase in "caries-free" children during the period, *especially* between 1943 and 1947.

These findings indicate that great improvements are actually taking place in the teeth of English children and it is impossible *to avoid the conclusion that the change* is largely due to the alterations in food supplies directed to

the improvement in calcium metabolism, which has been the subject of discussion in the second part of these lectures.

In view of the foregoing account of the anticalcifying action of cereals, it is to be noted that the present improvement in calcification processes in the population of Britain during and since the war has taken place in spite of the greater relative consumption of such foodstuffs, involving as it has, a higher intake of phytate. Here, then, is some evidence of a practical nature, taken from the life of the community, showing that the teachings based on the results of animal experiments described in the last two lectures, are correct, and that cereals with a high phytate content, although in themselves harmful and even dangerous to life, and especially early life, can be made innocuous by including in the diet other foods rich in calcium and containing vitamin D.

Final Remarks

In this series of lectures the two main lines of investigation that have been described and discussed had a common origin in experimental work on the cause of rickets. Although they started on their respective courses from different angles, one as a study of nervous defects and the other as a study of defects in calcification, they ultimately became focussed once more on closely related physiological functions of the same anatomical structures.

The earlier lectures demonstrated the fact that the nerve defects observed in some experimental animals resulted from pressure on or distortion of the nervous tissue by hyperplastic bone. This malformation of bone, which had such harmful effects, was found to be due to the loss of moulding resulting from a deficiency of vitamin A in the body. The control of bone shape by vitamin A was shown to be related to its power over the position and intensity

of activity of the osteoblasts and osteoclasts of appositional bone in the growing skeleton.

The second group of lectures, which dealt with the action of cereals in interfering with calcium metabolism and therefore with the hardening of bones, showed that this biochemical action was due to the phytate content of cereals and its interference under some conditions with the functional activity of vitamin D. Phytate and vitamin D have been seen to be natural enemies, the former trying to expel calcium from the body, and the latter promoting its absorption and retention. An attempt was made in these later lectures to show something of the nature of the fight between these two substances. It was demonstrated that, by its power to combine with calcium and form an insoluble compound in the alimentary canal, phytate was often able to reduce the amount of this element coming under the influence of vitamin D and so to prevent calcium from being absorbed and from fulfilling its proper function, especially in relation to bone and tooth formation. On the other hand, it was made clear that vitamin D normally controls the conversion of phytate to inorganic phosphate, probably by promoting its hydrolysis by a phytase, thus rendering much of it harmless.

Although, therefore, the problems dealt with in these lectures cover a much wider field, they can be regarded from one point of view as centering round bone formation, the vitamin A lectures being concerned with the shaping and modelling of bones and the phytate and vitamin D lectures with the hardening of these structures.

In the early days of this work (1919 to 1921) it was demonstrated that a fat-soluble vitamin controlled the calcification of bones and teeth. In course of time it became evident that the vitamin A of those days was a complex of two vitamins, one retaining the name vitamin A and the other now called vitamin D. The present lectures

have shown that these two entities work together in close association. First vitamin A controls, or at least influences, the activity of the osteoblasts which lay down the soft bone matrix. Vitamin D then attends to the calcification of this osteoid tissue, and finally vitamin A again steps in and sees that any superfluous calcified bone is removed by osteoclastic action and that the bone shape is correct.

Thus it has been seen that these two inseparables, vitamins A and D, the David and Jonathan of nutrition, whose faithful alliance in distribution and similarity of many chemical and physical properties has caused so much trouble to hosts of physiologists, biochemists and other scientists, work in harmony and on the same structures at the time of their active careers in the animal body. Although their functions are different, they unite in directing *and controlling the building up and maintenance of* bone structure.

of activity of the osteoblasts and osteoclasts of appositional bone in the growing skeleton.

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2. CHEMICAL TREATMENT OF SOME DIETS

(a) *Hydrolysis of phytate of oatmeal by boiling with HCl*

(Experiments 7, 8 and 12)

750 g. of oatmeal were mixed with dilute HCl so that the final concentration of acid was 1 per cent, heated on a water bath for 30 minutes and then transferred to a heated sand tray. For the next 30–45 minutes the mixture was stirred until it began to boil; boiling was then continued on an electric sand bath under a reflux condenser for the requisite period. The mass was next cooled under running water, the HCl was neutralised with NaOH, and the bulk was made up to 2250 ml.

(b) *Hydrolysis of phytate of oats by germination and autolysis*

(Experiments 9 and 10)

The method employed consisted of three stages—steeping, germination and autolysis of the germinated and minced grain.

Steeping. The oats were stood in distilled water in glass or enamel vessels for about 18 hours; the water was then removed and the grain drained for about 6 hours. This process was repeated twice more, using the same fluid for each immersion.

Germination. The swollen grain was then spread over enamel trays (layers about 1" thick) and placed in a large box within which a humid atmosphere (Temp. 25°C.) was maintained by means of a constant stream of warm moist air. The trays were examined daily, the grains being carefully stirred and any loss of water made good.

Autolysis After 8 days' germination the grain was minced and the mass returned to the warm cabinet for a further two days. The liquor used in steeping was then added to the minced and autolysed grain and the whole mass was boiled.

The untreated oats used in the control diets were ground and then stirred into boiling water; this ensured that the phytase of the oats was destroyed with the minimal hydrolysis of phytate.

(c) *Purified sodium phytate and Phytic acid from commercial phytin*

(Experiment 4)

The method of preparation was based on that described by Posternak (1921). To 2 l of $N/3$ HCl, 200 g of phytin were added and a small excess of ferric chloride (2 l. of 7% $FeCl_3$ solution)

Appendix II

EXPERIMENTAL METHODS

1. DIETS

Most of the litters used in this work were bred at the laboratories from bitches maintained on diets composed of ordinary foodstuffs, including cereals, whole milk powder, scrap meat and cod-liver oil. During gestation and lactation the dietary Ca was increased, usually by the addition of whole milk powder, so that the mothers had up to 800 mg. of Ca daily in their food and tap water.

The litters were given food independently of the mother from the 3rd or 4th week after birth, the diet until weaning, which was complete at 6 weeks of age, being of the following form: bread, separated milk powder (up to 25 g.), lean meat (up to 15 g.), compressed baker's yeast, NaCl, ascorbic acid and either peanut oil or cod-liver oil (see individual experiments). This diet was usually continued until the experimental diet was begun.

Full details of the experimental diets are given in the text, but the following general notes may be added here:

(1) The white flour was specially milled and no Ca, P or improver was added. There was little or no Ca in the water as in most of the experiments it was either distilled or specially softened.

(2) The Ca content of the basal experimental diets was low but was sufficient, when vitamin D was added, to ensure good calcification except when the diet had a high phytate content.

(3) The daily cereal intake increased as the puppies grew and the Ca:P ratio of the diet was therefore reduced as the experiment proceeded, but was comparable for all animals within an experiment unless otherwise arranged. The cereals were cooked in a pressure steamer (½ lb. pressure) for 1½ hours and the yeast was boiled with water before being added to them.

(4) All visible fat was removed from the meat.

(5) Various sources of vitamin A were used, but in the later experiments it was given only in the form of vitamin A acetate. The source of vitamin D was calciferol (irradiated ergosterol) and the amounts administered were expressed as international units (i.u.).

In experiments which were made to compare the effects of sodium phytate and inorganic phosphate, the latter was prepared from sodium phytate by hydrolysis with yeast (p. 266) A solution of Na phytate was mixed with a suspension of baker's yeast (25 mg. of P to 1 g. of yeast), the pH was corrected to 4 5 and the mixture was incubated at 45°C for 12 hours with constant stirring At the end of this period the solution was boiled for 5 minutes, cooled, and the HCl was neutralised with NaOH This treatment converted all the phytate to inorganic phosphate.

As controls, similar solutions and suspensions of Na phytate and yeast were incubated separately as above The yeast was boiled and when cool was added to the phytate solution, the HCl being neutralised as before This mixture contained the same amount of P as the previous one, but in the form of phytate, with only a trace of inorganic P.

(c) *Preparation of Ca phytate*

(*Experiment 17*)

4000 ml. of a solution containing 5 mg of P per ml as sodium phytate were corrected to pH 5 5 with HCl and 1000 cc of 6% $CaCl_2$ solution, also at pH 5.5, were added After being stirred briskly for a few minutes, the mass was filtered on a Buchner funnel, washed with a small quantity of water and then with 80% or 90% alcohol. The Ca phytate was then spread out to dry at room temperature and the Ca, total P and phytate P estimated.

3. ASSESSMENT OF RESULTS

The methods of assessing the experimental results included (1) X-ray examinations, (2) the estimation of A/R ratios, and (3) a study of the mineral metabolism balances In general these findings were supported by the appearance of the animals and the histological examination of the bones

As regards the X-ray appearances, an attempt has been made to number the stages of rickets from 1 to 10, but it must be pointed out that these numbers, although relative, do not represent absolute degrees. During life the diagnosis was made from X-rays of the left forepaw only, but post mortem the appearance of the ribs and other bones was considered.

The method of marking and collecting faecal and urine samples is given on p. 292. The collected faecal samples were heated to 100°C within 9 hours of collection, dried, weighed, ground and stored for future analysis of Ca, phytate P and total P.

was stirred in. The precipitate of iron phytate was filtered on a large Büchner funnel, suspended by mechanical stirring in 3 l. of N/6 HCl, filtered and thoroughly suspended in 2 l. of water. An excess of 40% NaOH (about 350 ml.) was added gradually and the mixture was stirred for ½–1 hour; sufficient 5N HCl (about 50 ml.) was then added to make the reaction just alkaline to thymolphthalein (about pH 9). 100 g. NaCl were added (to assist in the precipitation of the colloidal ferric hydroxide) and the mixture was filtered by suction. The filtrate was usually practically free from iron, though an occasional preparation required further treatment with acid or alkali or by warming in order to precipitate all the colloidal iron. The precipitate was washed by stirring with 1 l. of water containing 10 ml. of 40% NaOH and then brought back to about pH 9 and filtered, and the combined filtrate and washings were treated with half their volume of absolute alcohol and allowed to stand overnight in the refrigerator. The alcohol was poured off from the semi-crystalline alkaline sodium phytate, which was then washed with a little 33% (by vol.) alcohol, dissolved in 100 ml. hot distilled water, warmed in a dish on the water bath to drive off residual alcohol and made up to about 300 ml. with water. A small volume of the syrupy solution was measured out in a blood pipette for determination of phytin P by the method of McCance and Widdowson (1935). The solution usually contained the equivalent of 45–50 per cent anhydrous sodium phytate, and was practically free from inorganic P.

A portion of the solution was converted into free phytic acid by stirring in concentrated HCl until the solution was just acid to Topfer's indicator. To another portion HCl was added until it became neutral to phenol red. These solutions were used in Experiment 4. Both solutions were diluted before mixing each day with the previously cooked basal diet and were fed in quantities equivalent to the phytate P eaten by the oatmeal control puppy.

(d) Sodium phytate and sodium phosphate

(Experiments 11, 13, 14, 15, 16 and 19)

Sodium phytate was prepared from commercial phytin by the method described in (c) above, except that no NaCl was added and the sodium phytate solutions and washings were neutralised to phenol red before adding the alcohol. The sodium phytate formed a heavy, practically colourless, syrupy liquid containing no appreciable amount of inorganic or other non-phytate phosphorus. (Harrison and Mellanby, Biochem. J., 1939, 33, 1671.)

In experiments which were made to compare the effects of sodium phytate and inorganic phosphate, the latter was prepared from sodium phytate by hydrolysis with yeast (p. 266). A solution of Na phytate was mixed with a suspension of baker's yeast (25 mg. of P to 1 g. of yeast), the pH was corrected to 4.5 and the mixture was incubated at 45°C. for 12 hours with constant stirring. At the end of this period the solution was boiled for 5 minutes, cooled, and the HCl was neutralised with NaOH. This treatment converted all the phytate to inorganic phosphate.

As controls, similar solutions and suspensions of Na phytate and yeast were incubated separately as above. The yeast was boiled and when cool was added to the phytate solution, the HCl being neutralised as before. This mixture contained the same amount of P as the previous one, but in the form of phytate, with only a trace of inorganic P.

(e) *Preparation of Ca phytate*

(*Experiment 17*)

4000 ml. of a solution containing 5 mg. of P per ml. as sodium phytate were corrected to pH 5.5 with HCl and 1000 cc of 6% $CaCl_2$ solution, also at pH 5.5, were added. After being stirred briskly for a few minutes, the mass was filtered on a Büchner funnel, washed with a small quantity of water and then with 80% or 90% alcohol The Ca phytate was then spread out to dry at room temperature and the Ca, total P and phytate P estimated

3. ASSESSMENT OF RESULTS

The methods of assessing the experimental results included (1) X-ray examinations, (2) the estimation of A/R ratios, and (3) a study of the mineral metabolism balances In general these findings were supported by the appearance of the animals and the histological examination of the bones.

As regards the X ray appearances, an attempt has been made to number the stages of rickets from 1 to 10, but it must be pointed out that these numbers, although relative, do not represent absolute degrees During life the diagnosis was made from X rays of the left forepaw only, but post mortem the appearance of the ribs and other bones was considered.

The method of marking and collecting faecal and urine samples is given on p. 292 The collected faecal samples were heated to 100°C within 9 hours of collection, dried, weighed, ground and stored for future analysis of Ca, phytate P and total P.

was stirred in. The precipitate of iron phytate was filtered on a large Büchner funnel, suspended by mechanical stirring in 3 l. of $N/6$ HCl, filtered and thoroughly suspended in 2 l. of water. An excess of 40% NaOH (about 350 ml.) was added gradually and the mixture was stirred for ½–1 hour; sufficient 5N HCl (about 50 ml.) was then added to make the reaction just alkaline to thymolphthalein (about pH 9). 100 g. NaCl were added (to assist in the precipitation of the colloidal ferric hydroxide) and the mixture was filtered by suction. The filtrate was usually practically free from iron, though an occasional preparation required further treatment with acid or alkali or by warming in order to precipitate all the colloidal iron. The precipitate was washed by stirring with 1 l of water containing 10 ml. of 40% NaOH and then brought back to about pH 9 and filtered, and the combined filtrate and washings were treated with half their volume of absolute alcohol and allowed to stand overnight in the refrigerator. The alcohol was poured off from the semi-crystalline alkaline sodium phytate, which was then washed with a little 33% (by vol.) alcohol, dissolved in 100 ml. hot distilled water, warmed in a dish on the water bath to drive off residual alcohol and made up to about 300 ml. with water. A small volume of the syrupy solution was measured out in a blood pipette for determination of phytin P by the method of McCance and Widdowson (1935). The solution usually contained the equivalent of 45–50 per cent anhydrous sodium phytate, and was practically free from inorganic P.

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The methods used in the analysis were:

(1) *Estimation of phytate phosphorus* in foods, faeces and urine was carried out by the method of McCance and Widdowson (1935), with slight modifications, including an increase in the concentration of the ferric chloride solution.

(2) *Total phosphorus* in foods, faeces and urine was estimated by the method of McCance and Widdowson (1935).

(3) *Inorganic phosphorus* and the *total and phytate phosphorus after incineration* were estimated colorimetrically by various methods, including those of Briggs (1922), Fiske and Subbarrow (1925), and Allen (1940). For the final comparisons a Spekker photoelectric absorptiometer was used.

(4) *Calcium in foods, faeces and urine* was estimated by various methods, principally those used by Macy (1942), McCance and Widdowson (1942a) and, for foodstuffs, the Imperial Bureau of Animal Nutrition (1937).

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